Studies of UHPLC-MS performance and application to rapid sensitive and robust drug analysis

Gray, Nicola

Awarding institution:
King's College London

STUDIES OF UHPLC-MS PERFORMANCE AND APPLICATION TO RAPID SENSITIVE AND ROBUST DRUG ANALYSIS

NICOLA GRAY

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

ANALYTICAL AND ENVIRONMENTAL SCIENCE
KING'S COLLEGE LONDON

2013

KING'S COLLEGE LONDON

FRANKLIN WILKINS BUILDING

150 STAMFORD STREET

LONDON SE1 9NH

SUPERVISORS:

DR. NORMAN W SMITH

PROF. DAVID A COWAN

ABSTRACT

This thesis comprises studies of ultra high performance liquid chromatography (UHPLC) performance with a particular focus on the application of liquid chromatography-mass spectrometry (LC-MS) for the analysis of basic drugs. UHPLC-MS technologies are investigated to facilitate fast, sensitive and robust analysis of drugs in biological matrices, particularly those related to doping in human sport.

Mobile phase solvent, pH and temperature significantly affect the chromatographic performance of hydrophilic, basic analytes. Manipulation of mobile phase pH suppresses the protonation of basic analytes for improved retention, peak shape and resolution. This is exemplified by the ephedrines, which are inherently difficult to separate by reversed-phase liquid chromatography (RPLC). A high pH RPLC separation is coupled with MS detection and validated for the identification and quantification of ephedrines in doping control analysis. The effects of mobile phase composition and pH on efficient and stable ionisation are studied for robust and sensitive analysis of basic analytes. Different mobile phase conditions are evaluated with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), with high pH eluents generating greater signal intensities for the basic analytes studied with ESI.

Hydrophilic interaction liquid chromatography (HILIC) is evaluated as an alternative approach for accurate and robust quantification. A HILIC approach to separate the ephedrines is evaluated for robustness, and the validated method is

compared with the high pH RPLC method in terms of linearity, accuracy, precision, matrix effects and sensitivity.

Finally, a switching system comprising two different, complementary stationary phase materials is designed and evaluated to widen the elution window, allowing for the simultaneous analysis of both polar and non-polar analytes. A combination of Hypercarb and C_{18} stationary phases are used with UHPLC column switching, and the suitability of the approach is illustrated by the analysis of selected doping agents covering a wide polarity range in a single injection.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors Dr. Norman Smith and Prof. David Cowan for all of their help and guidance over the past four years. It has been a privilege to work with such experts from whom I have learnt so much. I am also grateful to Prof. Robert Plumb and Waters Corporation (Milford, USA) for the funding and support of this PhD research.

I am grateful to Dr. James Heaton (King's College London) and Dr. Alessandro Musenga (Drug Control Centre) for their contribution to collaborative work and from whom I have learnt a great deal about liquid chromatography and mass spectrometry. Thanks are also extended to Michael Jones and Paul Rainville (Waters Corporation, USA) for their help and to Dr. Bob Boughtflower (GSK, Stevenage) for collaborating with us.

The colleagues I have worked with at King's College and friendships I have made during my time here have made this experience so enjoyable. I thank all everyone from the Microseparations Group, Drug Control Centre and fellow PhD students, with a special mention to Luke Whiley, James Heaton and Fred Warren.

I would like to thank all of my friends, but especially Vicky Hoof for her help which has been invaluable. Last but not least, I thank my family, and in particular my parents, for their continued support and encouragement.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Selectivity factor
η	Mobile phase viscosity
λ	Column packing factor
γ	Obstruction factor
ψ	Association factor
$\varnothing$	Phase ratio
µg	Microgram
µL	Microlitre
µm	Micrometre
A	Eddy diffusion coefficient
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
A_s	Asymmetry factor
B	Longitudinal diffusion coefficient
BEH	Bridged-ethyl hybrid
C	Mass transfer coefficient
°C	Degrees Celsius
CH_3CN	Acetonitrile
C_3H_8O	Propan-2-ol
CH_3OH	Methanol
CHOOH	Formic acid
cm	Centimetre
cP	Centipoise
d_p	particle diameter
D_m	Diffusion coefficient of analyte in mobile phase
D_s	Diffusion coefficient of analyte in stationary phase
Eph	Ephedrine
ESI	Electrospray ionisation
FWHM	Full width at half maximum
g	G-force
GC	Gas chromatography

ΔH_0	Enthalpy of solute transfer
FWHM	Full width at half maximum
h	Peak height
H/*HETP*	Height equivalent to theoretical plate
H_{min}	Minimum plate height
HILIC	Hydrophilic liquid interaction liquid chromatography
HPLC	High pressure liquid chromatography
HRAMS	High resolution accurate mass spectrometry
k	Retention factor
K	Kelvin
K_a	Acidity constant for an acid or base
K_{v0}	Column permeability
L	Length
LC-MS	Liquid chromatography-mass spectrometry
log$D$	Distribution coefficient
log$P$	Partition coefficient
Meph	Methylephedrine
mg	Milligram
mL	Millilitre
mm	Millimetre
min	Minute
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
N	Number of theoretical plates
NH_4OH	Ammonium hydroxide
NPD	Nitrogen phosphorous detector
ng	Nanogram
ΔP	Pressure drop
Pa	Pascal
PCG	Porous graphitic carbon
Peph	Pseudoephedrine
pK_a	logarithm of the acidity constant for an acid or base

PPA	Phenylpropanolamine
psi	Pounds per square inch
QQQ	Tandem (triple) quadrupole
QTOF	Quadrupole time-of-flight
R	Universal gas constant
R_s	Resolution
RPLC	Reversed-phase liquid chromatography
ΔS_0	Entropy of solute transfer
SRM	Selected reaction monitoring
T	Temperature
t_o	Retention time of an unretained peak (dwell time)
t_R	Retention time
TEA	Triethylamine
TFA	Trifluoroacetic acid
TIC	Total ion count
TOF	Time-of-flight
u	Linear velocity
u_{opt}	Optimum linear velocity
UHPLC	Ultra high pressure liquid chromatography
UPLC®	Ultra high pressure liquid chromatography® (Waters Corporation)
UV	Ultraviolet absorption
V_A	Molar volume
v/v	Volume per volume
v_o	Equipment dwell volume
w_b	Peak width at base
$w_{1/2}$	Peak width at half height
WADA	World Anti-Doping Agency
w/w	Weight per weight
XIC	Extracted ion chromatogram

CHAPTER 1

INTRODUCTION

1.1 Introduction to Liquid Chromatography-Mass Spectrometry (LC-MS)

1.1.1 Advances in LC-MS

Rapid advances in liquid chromatography and mass spectrometry have enabled the true benefits of the hyphenated technique (LC-MS) to be realised over the past decade. In the first place, significant improvements in chromatographic performance have been achieved in terms of analysis speed, resolving power and sensitivity as a consequence of reduced particle diameter and the advent of ultra-high pressure pumping systems. The influence of reducing particle size on column efficiency has been understood for many years. The van Deemter equation describes the relationship between column performance and particle size due to shorter diffusion distances. Since the introduction of HPLC, there has therefore been a continued trend in reducing particle size, from traditional 10 µm particles, down to 5 µm, 3 µm and more recently sub-2 µm. However, the use of smaller particle diameters comes at the expense of operating pressure, which is inversely proportional to the square of the particle size. The first reports of using very small particles (1.5 µm nonporous particles) and ultra-high pressures (59,000 psi) were published by MacNair *et al.* in 1997 [1, 2]. The use very small particles (sub-2 µm) was therefore limited by the capability of conventional system pumps and autosamplers, not able to withstand ultra-higher pressure conditions. Additionally, the ability to operate at the elevated pressures associated with the reduction in particle diameter demands the technology to manufacture and pack particulate structures that are able to tolerate ultra-high pressures [3, 4]. A further consideration is the need for low system volume when migrating to the smaller column dimensions used with reduced particle diameter, where contributions from extra-column band broadening become paramount [5].

The evolution of UHPLC has been driven by the demand for faster separations and the need to separate more and more compounds in complex matrices [4]. Chromatographic resolution is influenced by several factors, but the most significant is plate count. It is therefore possible to achieve greater resolution with longer columns, although this has a negative impact on the speed of analysis and hence sample throughput. Consequently, various approaches have been used to achieve fast LC separations without sacrificing resolution. High-temperature LC has been used to reduce mobile phase viscosity, which enables the chromatographer to use longer column formats and increase flow rate while still working with the pressure limits of the system for a gain in resolution [6, 7]. Other approaches in column technology include the use of monoliths [8, 9] and core-shell particles [10-12]. Both techniques offer shorter diffusion distances and improved mass transfer, but without the inherently large back-pressures of small porous particles. While the commercial availability of monoliths remains limited, core-shell particles have been shown to be a promising alternative to sub-2 µm materials, offering similar gains in efficiency and resolution without requiring expensive, dedicated instrumentation [13-15]. Nevertheless, the advent of UHPLC technology has provided the most widespread and well-established approach, based on the use of sub-2 µm particles operated at ultra-high pressures (up to 15,000 psi). The extended pressure range of UHPLC instrumentation enables the chromatographic potential of sub-2 µm particles to be harnessed, providing significant improvements to analysis time, resolution and sensitivity [16-19]. The considerable developments in the evolution of UHPLC instrumentation over the past decade means the use of sub-2 µm particle materials and operation at pressures up to 15,000 psi is now becoming commonplace in many analytical laboratories. However, highly efficient UHPLC separations generate

inherently narrow peak widths (1-3 sec), demanding a fast scanning detector to record a sufficient number of data points for accurate peak definition (at least 15 for quantification).

Mass spectrometry (MS) is the most commonly used detection technique with UHPLC for the rapid analysis of compounds present at low concentrations in complex matrices (employed in over 60 % of applications), combining sensitivity, selectivity and unambiguous identification [20]. It follows, therefore, that mass spectrometric technologies have had to evolve to enable fast acquisition rates to garner the benefits afforded by UHPLC. Tandem triple quadrupole MS remains the dominant approach for targeted screening, identification and quantification of small molecules in bioanalysis, providing unrivalled selectivity and sensitivity [21]. The need for fast scanning data capture when hyphenating UHPLC with MS has therefore resulted in the commercialisation of modern triple quadrupole mass analysers with reduced dwell times (5 ms) for improved acquisition rates. However, the past few years have seen a significant shift towards the use of high resolution accurate mass (HRAM) instrumentation coupled with UHPLC for targeted screening and even quantification assays [22]. Advances in MS technologies have resulted in HRAM instrumentation capable of very fast acquisition rates, which are also able to achieve acceptable sensitivity and the dynamic range required for quantification [23, 24]. One example is the availability of improved time-of-flight (TOF) and hybrid quadrupole-TOF (QTOF) analysers, providing medium resolution and accurate mass measurements, which present an attractive alternative to triple quadrupole analysers for hyphenation with UHPLC, particularly for high-throughput screening assays [25-28].

1.1.2 Application of LC-MS to bioanalysis

There is a continued need in bioanalysis for more comprehensive, sensitive and faster detection tools, driving the improvement or development of new drug testing procedures. Such is the case in doping control analysis, with an ever-growing number and variety of substances to test for, and with restrictions on speed and cost of analysis, comprehensive, high-throughput assays are highly sought after. UHPLC offers the high resolution required to separate structurally related compounds from endogenous components in complex sample matrices, such as urine and plasma, and offers faster separations without sacrificing resolution. In addition, the increase in sensitivity with narrow peaks widths generated by less resistance to mass transfer, improve detection limits and in some cases permit longer detection times. As well as the importance of analysis time, bioanalytical assays must also meet rigorous criteria in selectivity, specificity, accuracy, precision and linearity which necessitates suitable sample preparation and validation to ensure robust, accurate methodologies.

In the bioanalytical arena, gas-chromatography (GC) has long been established as the "gold standard" for the separation of small molecules. However, with the recent developments in LC-MS, this approach has become increasingly important for robust and reliable assays in the fields of clinical and forensic toxicology and doping control [29-33]. LC has become an attractive alternative to GC, with the ability to analyse hydrophilic, thermolabile and non-volatile analytes, which were not sufficiently covered by GC. Additionally, LC-MS offers a fast, sensitive and selective approach without the need for derivatisation. Omitting this step which can be labour-intensive and time-consuming is a vital advantage of LC, where the simple sample preparation is amenable to fast, high-throughput assays and limits potential

sources of error. This is particularly important considering the improvements to separation speed with UHPLC, where the limiting factor in total analysis time now lies in sample preparation.

The flexibility of LC coupled with various types of MS detector has permitted qualitative and quantitative analysis, providing highly selective and sensitive assays capable of analysing a wide range of analytes from a variety of sample matrices. Consequently, LC-MS has become widely adopted as the leading technology in a variety of applications. For example, the field of doping control analysis is just one example of where fast, high-throughput methodologies are required, especially at large international events, where turn-around times can be as little as 24 hours. Typically, the analytical workflow comprises an initial generic screen to eliminate negative samples as early as possible, while any suspect sample is subjected to more rigorous, targeted analysis for identification and, where necessary, quantification.

1.2 Theory of Liquid Chromatography

1.2.1 Introduction to High Performance Liquid Chromatography (HPLC)

Chromatography can be defined as a physical separation technique whereby the components to be separated are distributed between two phases, one of which is stationary (*stationary phase*) while the other (the *mobile phase*) moves in a definite direction [34]. First realised by Twsett in the early 1900s, chromatography has since evolved into the modern technique now referred to as high performance liquid chromatography (HPLC). Since its development in the 1960s, HPLC has become

one of the most powerful separation techniques in analytical chemistry. An illustration of the components of HPLC system is given in Figure 1.1.

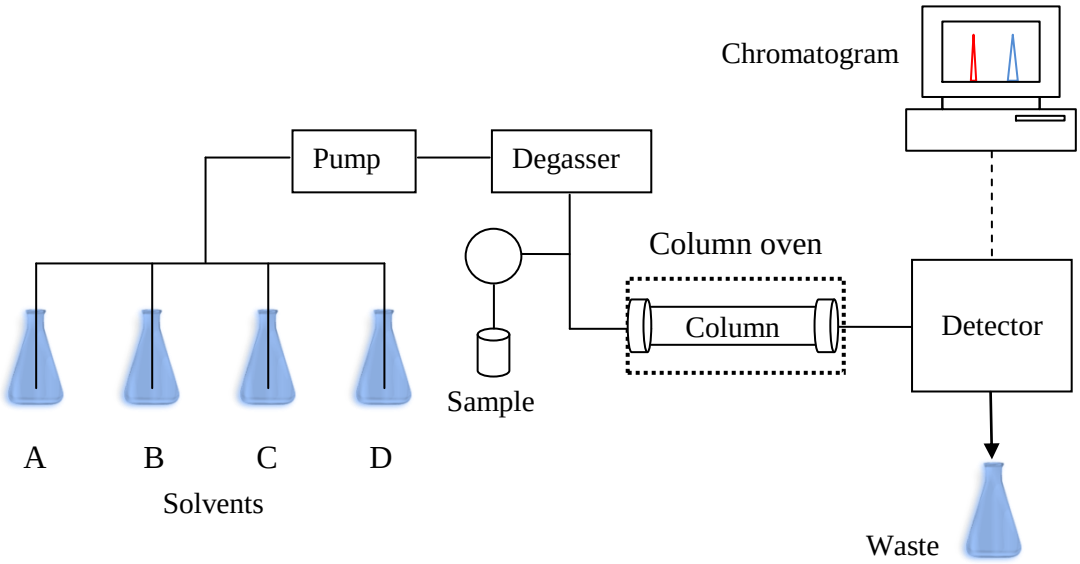


Figure 1.1. Components of a HPLC system.

In LC, a sample is separated into its individual components by distribution between a fixed stationary phase and a mobile phase. Differences in the chemical and physical properties of each component determine the affinity for the stationary and mobile phases and so influence the degree of separation. The greater the affinity a solute has for a particular stationary phase, the longer it will be retained and hence the later it will elute. This concept is described in Figure 1.2 below.

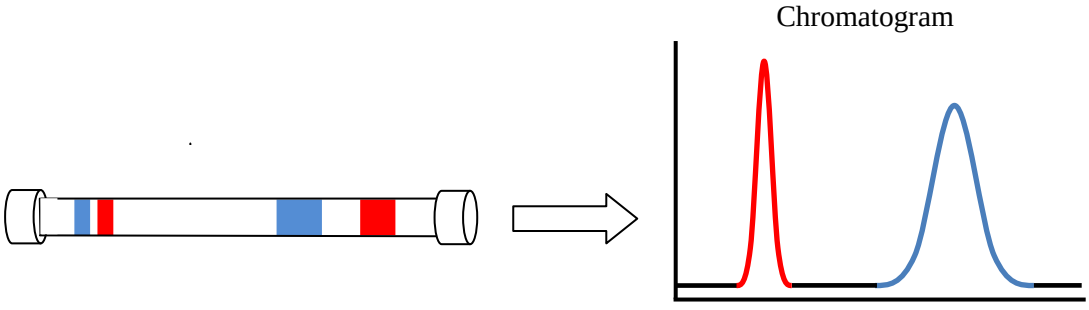


Figure 1.2. Representation of separation by liquid chromatography.

1.2.2 Chromatographic parameters

The detector in Figure 1.1 converts the digital information gained from the chemical separation of analytes into a visual form (chromatogram). Several important parameters can be determined from the chromatogram, useful in identifying and quantifying the components present. These are described in Figure 1.3 below:

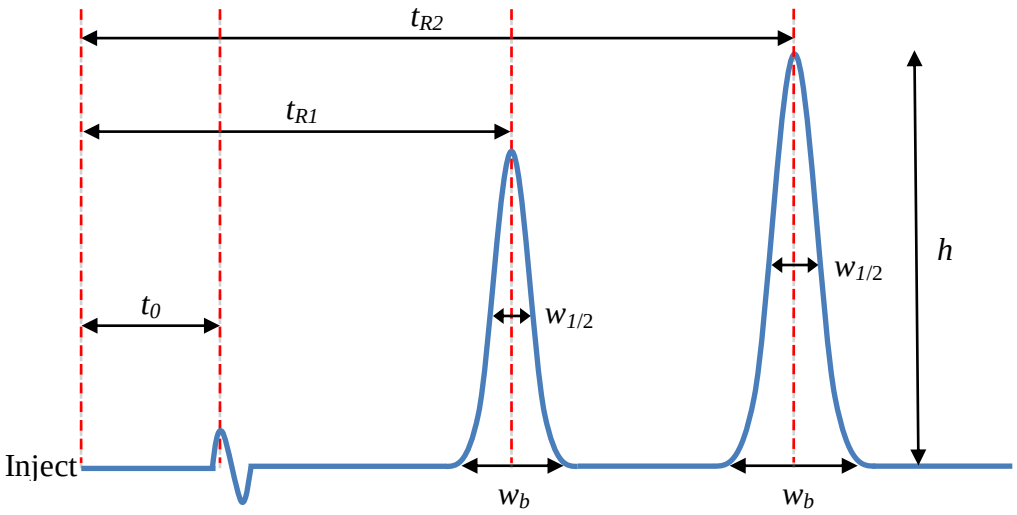


Figure 1.3. Chromatographic parameters.
A chromatogram showing void time (t_0), retention time (t_R), peak width (w_b), peak width at half height ($w_{1/2}$) and peak height (h).

1.2.2.1 Void time/volume (t_0/v_0)

The first peak, or disturbance in the baseline of a chromatogram, represents the retention time of an unretained solute and indicates the void time (t_0) or dead volume (v_0). This is the time taken for the mobile phase to reach the column from the point of injection and therefore is the time spent by any component in the mobile phase.

1.2.2.2 Retention time (t_R)

The retention time (t_R) of a component is the time taken from injection of the sample to the peak maximum. The void time (t_0) indicates the time spent by any component in the mobile phase, whereas t_R is the total time the solute spends in the stationary phase and in the mobile phase. The retention time can be adjusted to give the time spent by the solute in the stationary phase:

$$\boldsymbol{t'_R = t_R - t_0}$$

1.2.2.3 Retention factor (k)

Retention, or capacity, factor can be defined as the degree of retention of an analyte relative to an unretained peak, calculated by:

$$\boldsymbol{k = \frac{t_R - t_0}{t_0}}$$

where t_R is the retention time of the sample peak and t_0 is the retention time of an unretained peak.

Measuring retention factors will help determine whether retention shifts are due to the column (*k* changes with retention time) or the system (*k* remains constant with retention time changes).

1.2.2.4 Selectivity factor (α)

The selectivity factor (α) is a measure of the difference in retention of two substrates in a separation, expressed as:

$$\alpha = \frac{t_{2-}t_0}{t_1-t_0} = \frac{v_{2-}v_0}{v_1-v_0} = \frac{k_2}{k_1}$$

where k_1 and k_2 are respective retention factors.

Selectivity values must be > 1.0 for peak separation, a value of less than 1 illustrates no difference in the selectivity of analytes and therefore no separation between the two analytes. The selectivity is a function of column packing material and can be influenced by factors such as the mobile phase composition and temperature.

1.2.2.5 Resolution (Rs)

Resolution describes the degree of separation of two components, illustrating the ability of a column to separate chromatographic peaks. The resolution between two peaks in a separation can be measured by using the following equation:

$$R_s = \frac{(t_{R_2} - t_{R_1})}{0.5(w_{b_1} + w_{b_2})}$$

where t_R is the retention time of the sample peak, and w_b is the peak width at base, illustrated in Figure 1.4.

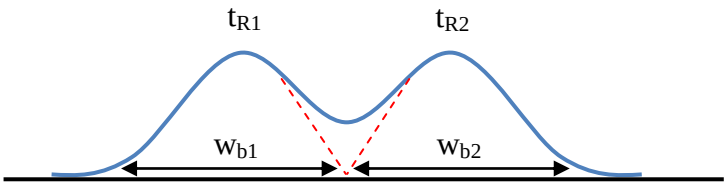


Figure 1.4. Illustration of peak resolution parameters.

A value of 1.5 typically indicates complete separation, although this assumes Gaussian peak shape and does not consider that one peak may correspond to more than one analyte. The boundaries between the baseline and the beginning and end of the peak are not well defined and depend on the threshold of the peak detection algorithm. For a Gaussian peak, the width of the peak is approximately equal to four times the standard deviation of the peak width (4σ) at the point if inflection which encompasses approximately 95 % of the peak area [35, 36].

1.2.2.6 Peak Symmetry: Asymmetry Factor (A_s)

Under ideal conditions, chromatographic peaks should be symmetrical, but in reality most exhibit either fronting or tailing, as depicted in Figure 1.5. Peak tailing is more common, resulting in lower peak heights and therefore higher detection limits, and also gives rise to difficulty in integrating peaks leading to imprecise and irreproducible peak areas. Determining where the limits of peak integration are to be located is ambiguous, a problem that is exaggerated with large tailing factors, thus causing errors in quantification. This is of particular importance where there is considerable difference in the size of two peaks which elute close together. A small peak which elutes after a much bigger peak with a large tailing factor may be obscured, especially if background noise is high.

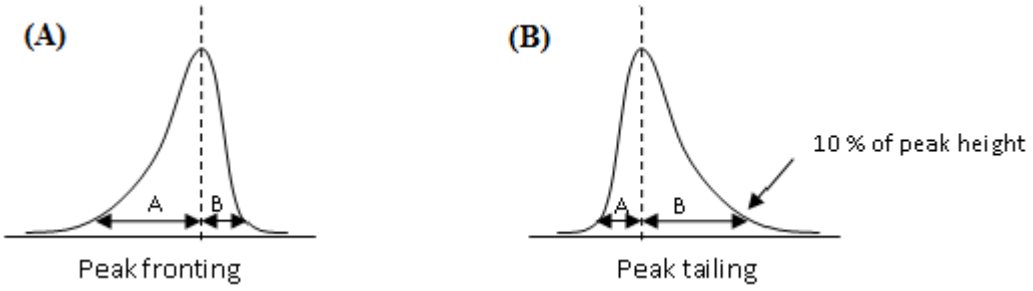


Figure 1.5. Illustration of peak fronting (A) and peak tailing (B).

The most commonly used method to quantify peak symmetry is the asymmetry factor (A_s), typically calculated using the peak width at 10 % of peak height:

$$\boldsymbol{A_s = \frac{B}{A}}$$

A value of 1 indicates a perfectly symmetrical peak, whereas a figure < 1.0 is a fronting peak and > 1.0 is a tailing peak. Peak tailing may result from several factors, including, band broadening and secondary interactions experienced between basic analytes and residual silanol groups on silica-based columns.

1.2.2.7 Column efficiency/plate number (N)

Also known as the number of theoretical plates, *N* is a measure of the quality of a separation that is based on a single peak and can be determined by various methods.

$$\boldsymbol{N = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2}$$

where *N* is the number of theoretical plates, t_R is the retention time, w_b is the peak width at base and $w_{1/2}$ is the peak width at half height.

Plate count is determined from the chromatogram and is a dimensionless parameter; it can be obtained from the peak width and retention time, provided that the migration velocity of the peak has been constant (isocratic chromatograms). Such a measurement, therefore, takes into account only peak dispersion, unlike resolution which is based on the distance between two peaks and their dispersion.

1.2.2.8 Height Equivalent to a Theoretical Plate (HETP)

The height equivalent to a theoretical plate (*HETP* or *H*) is a measure of a column's efficiency and can be expressed as:

$$\boldsymbol{HETP = \frac{L}{N}}$$

where *L* is column length, in millimetres, and *N* is the number of theoretical plates.

The theoretical plate concept originates from the distillation theory of gas chromatography which describes the number of plates in an industrial distillation column. Hence, the more plates per column, the better the separation. However, since the *HETP* is a function of many different parameters, the plate count is not a basic property of a column and therefore not a measure of column quality.

1.2.3 Theory of Band Broadening

Upon injection, an analyte forms a narrow band on the top of a column, which broadens as the analyte migrates through a chromatographic system and becomes diluted in the mobile phase. The longer an analyte takes to travel through a column, the greater the spread of individual molecules and the wider the band, resulting in a broader chromatographic peak and less efficient separation. The extent of band dispersion is dependent upon several contributing factors – those from the column itself and extra-column sources which may broaden or distort peak shape, including the injector, connection tubing and detector.

The van Deemter equation explains band broadening in chromatography due to the migration of an analyte through the column. The equation combines the individual sources of band broadening and represents them as the dependence of height equivalent to a theoretical plate (*HETP*) on the mobile phase linear velocity (*u*).

1.2.4 Van Deemter Equation

The van Deemter equation describes the dependence of plate height (*H*) on linear flow velocity (*u*), illustrating the relationship between the three different, independent contributions:

$$\boldsymbol{H = A + \frac{B}{u} + Cu}$$

where *A* represents the contribution of eddy diffusion, *B* represents the contribution from longitudinal or axial diffusion and *C* represents the contributions of mass transfer in the stationary and mobile phase.

1.2.4.1 Eddy Diffusion

The eddy diffusion (*A*) term describes the multiple path dispersion of the individual solute molecules as they travel through a packed column; some molecules of the same compound will take longer paths while others, for instance those closer to the walls of the column, will take more direct paths and therefore elute first (Figure 1.6). This is a velocity-independent term which is a function of particle size and the distribution of interparticle channels and other non-uniformities in the packed bed; the *A* term is proportional to d_p and is smaller in a well-packed column. A narrow particle size distribution reduces the effects of eddy diffusion since the interstitial space between particles is virtually homogenous, resulting in high column efficiencies. Smaller particles reduce the interstitial space between particles and therefore allow more uniform and higher quality packing. Variance contribution from multi-path dispersion to the overall variance per unit length of the column:

$$\boldsymbol{A = 2\lambda d_p}$$

where λ is a the packing factor, and d_p is the particle diameter.

The theory of eddy diffusion assumes that the solute molecules remain in one flow stream, however, molecules can move laterally from one flow stream to another (lateral diffusion) where the velocity may be different, as shown Figure 1.6.

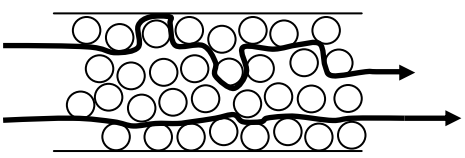


Figure 1.6. Illustration of Eddy diffusion.

1.2.4.2 Longitudinal Diffusion

Longitudinal or axial diffusion (*B* term) is a result of natural diffusion of the solute molecules in the mobile phase along the column axis, leading to broadening of the chromatographic zone. The extent of this diffusion is dependent on the time spent by the solute in the column and the diffusion coefficient of the compound in the mobile phase. The longer a solute remains on the column, the greater the extent of axial diffusion, as illustrated in Figure 1.7. The time a solute remains in the column is inversely proportional to the linear velocity of the mobile phase, so it follows that the dispersion will be inversely proportional to the linear velocity. The variance contribution by longitudinal diffusion can be expressed as:

$$\frac{\boldsymbol{B}}{\boldsymbol{u}} = \frac{\boldsymbol{2\gamma D_m}}{\boldsymbol{u}}$$

where γ is an obstruction factor which allows for restriction due to the packed particle, D_m is the diffusion coefficient of the solute in the mobile phase and u is the linear velocity of the mobile phase.

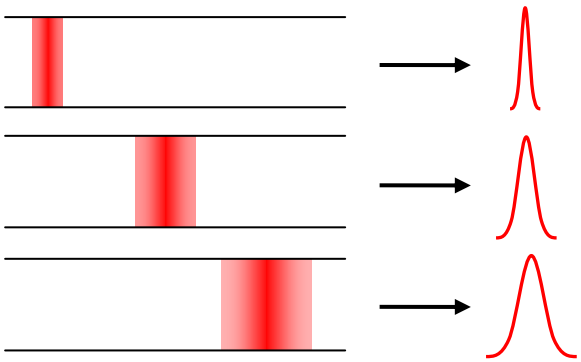


Figure 1.7. Illustration of longitudinal diffusion.

1.2.4.3 Resistance to Mass Transfer

Resistance to mass transfer (*C* term) is the most important factor affecting column efficiency and can be divided into the resistance to mass transfer of a molecule in both the mobile phase and the stationary phase. During migration through a chromatographic column, the solute molecules are constantly diffusing between the mobile phase and the stationary phase. Inefficient attainment of equilibrium between the stationary and mobile phases because of mobile phase flow is the cause of such contributions.

The resistance to mass transfer in the stationary phase is more or less the time needed for molecules to diffuse from the mobile phase into the stationary phase and back again. Differences in the depth of diffusion of solutes into the stationary phase results in a variable delay in the diffusion back into the mobile phase. The further into the stationary phase an analyte has diffused, the longer distance it will have to diffuse back into the mobile phase and the slower the rate of transfer compared with those that are close to the surface of the stationary phase.

The resistance to mass transfer in the mobile phase is divided into moving and stagnant mobile phase mass transfer. Contributions from the moving mobile phase result from molecules in the same flow path moving at different speeds. The solute molecules that are close to the particle or column wall will move slower than those in the bulk of the stream, causing band dispersion. This is dependent on the eddy diffusion, which needs to be coupled to the moving mobile phase mass transfer, and lateral diffusion, with slow lateral diffusion increasing band broadening.

The diffusion of solute molecules through the mobile phase to the interface of the stationary phase is not instantaneous; it takes time for the molecules to diffuse through the mobile phase to the stationary phase. Molecules close to the stationary phase will diffuse into it much more quickly than those some distance away, and while the mobile phase is moving they are continually swept along the column and move away from the molecules that were close and entered the stationary phase immediately. Thus, molecules which were originally relatively close together are not spread out in the stationary phase.

There are also contributions from the stagnant mobile phase situated in the pores of the packing material. Analytes that travel from the centre of the moving mobile phase to the surface of the particle must travel through the stagnant mobile phase in the pores to the stationary phase on the internal surface of the packing. This stagnant mobile phase reduces the distribution rate of the solutes between the stationary and mobile phases. Molecules that only diffuse a short distance into the pores will catch up with the bulk mobile phase more quickly than the ones diffusing further into the pores, causing band broadening.

The combined contribution from mobile phase mass transfer is given by:

$$\boldsymbol{Cu} = \frac{\boldsymbol{\lambda\, u\, d_p^2}}{\boldsymbol{D_m}}$$

where λ is the packing factor which allows for restriction due to the packed particle, u is the linear velocity of the mobile phase, d_p^2 is the particle diameter and D_m is the diffusion coefficient of the solute in the mobile phase.

A typical van Deemter curve (*HETP* vs *u*), as shown in Figure 1.8 below, illustrates the point at which the total dispersion from the three effects produces a minimum (H_{min}), giving the optimum linear velocity (u_{opt}) and flow rate of the mobile phase. Since the lower the *HETP* value the higher the plate number and the more efficient the column, the optimum flow rate is that where column efficiency is best.

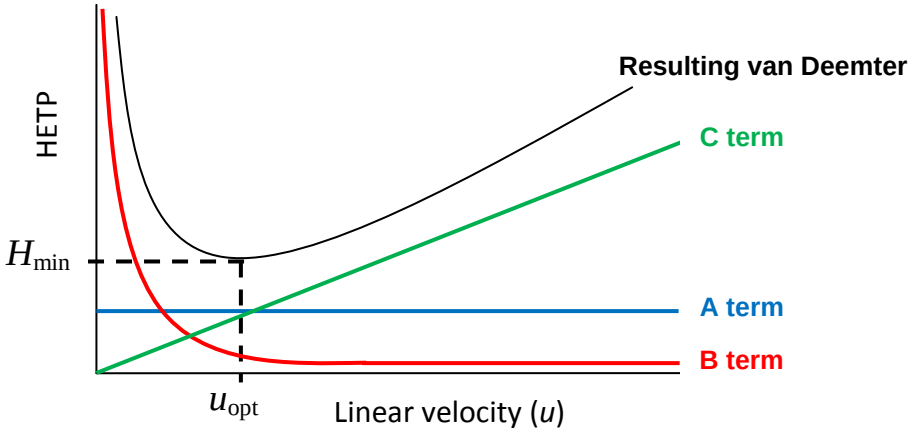


Figure 1.8. Typical van Deemter curve showing contribution from three dispersion mechanisms.

The faster the process of mass transfer, the better the efficiency of the column. Using smaller particulate packing material, less stationary phase, a less viscous mobile phase or higher temperatures can increase mass transfer. The effect of particle size on the van Deemter curve is illustrated in Figure 1.9, and it is this theory which has driven the reduction in particle diameter and the introduction of UHPLC hardware to support their operation over recent years [4].

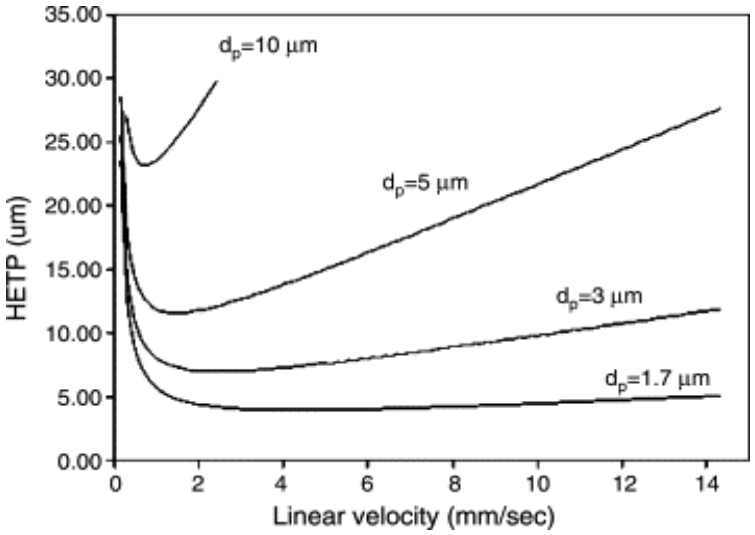


Figure 1.9. Van Deemter curves for different particle sizes (10, 5, 3 and 1.7 µm) [4].

1.2.5 Extra-column Effects

In addition to the dispersion contributions to band broadening from the column, described above, several extra-column factors can have a significant effect on band spreading and therefore the performance of a chromatographic separation [37]. Extra-column sources of band broadening include the injector, connection tubing and detection. The detrimental effects to band broadening caused by such extra-column factors become more apparent when using smaller i.d. columns (< 2.1 mm), smaller particles (< 3 µm) and smaller injection volumes which place a higher demand on the HPLC system [5]. In order to realise the potential of these columns, modified or specially designed instruments which minimise extra-column effects are necessary.

Since all of these dispersion processes can be independent, each variance can be added to obtain the total variance of a peak:

$$\sigma^2{}_t = \sigma^2{}_i + \sigma^2{}_f + \sigma^2{}_c + \sigma^2{}_d$$

where *i* is the band spreading in the injector and caused by injection volume, *f* is the band spreading in the fluid path between injector and detector, *c* is the band broadening inside the column and *d* is the band spreading caused by the detector.

1.2.6 Kinetic Performance Plots

With the evolution of LC columns, particles and particle sizes in recent years, there has been an increased desire to compare different formats with one another. Although the classical plate height plot (H, u_0) is useful for comparing phases of the same particle diameter, they do not consider the pressure drop, permeability or the time taken to realise a given number of theoretical plates. Consequently, kinetic plots have become a popular tool by which different supports can be compared. The basic kinetic plot of N versus analysis time (min), first used by Giddings in 1965 [38], has frequently been used to explain column performance and obtain information on the compromise between the analysis time and plate number that can be achieved for a given particle size and pressure limit. An example is given in Figure 1.10 which is used to compare kinetic plots for different particle sizes. It can be seen that, for short analysis times, the 2.5 µm particle always outperforms the 3.5 and 5 µm columns. However, with longer analysis times, the largest particle size provides the highest performance. This can be explained by the fact that for larger particles the diffusion-

controlled section of the kinetic plot is reached at a longer analysis time than for smaller particles.

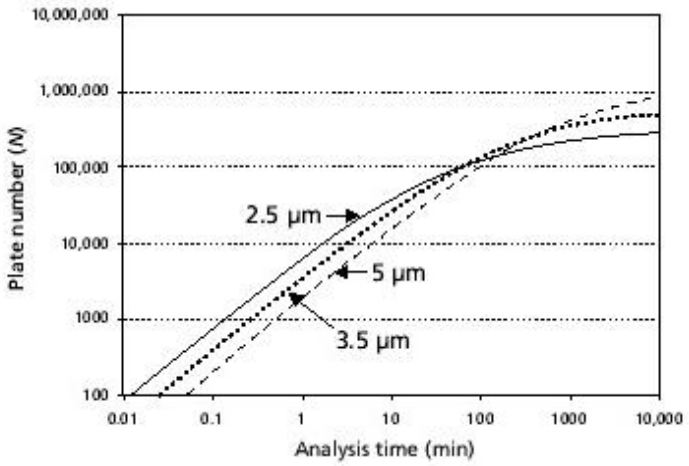


Figure 1.10. Kinetic plots of plate number versus analysis time.
Example of a plot of plate number versus analysis time for 2.5, 3.5 and 5 µm particles [39].

This simple plot was modified by Poppe who introduced the concept of plate time, the time required to generate one theoretical plate [40]. The t_R versus N plot can be easily converted into a Poppe plot by first inverting the axis to give the plate number (N) on the x-axis and the time on the y-axis. The analysis time is also replaced with the retention time of an unretained peak, the column dead time (t_0). This plot of t_0 versus N, as shown in Figure 1.11 (A), is commonly used to compare column performance under different chromatographic conditions. Dividing the dead time by the plate number to obtain the plate time (t_0/N) and plotting against N gives the classical Poppe plot that shows the highest achievable plate number for a given particle size, as illustrated in Figure 1.11 (B).

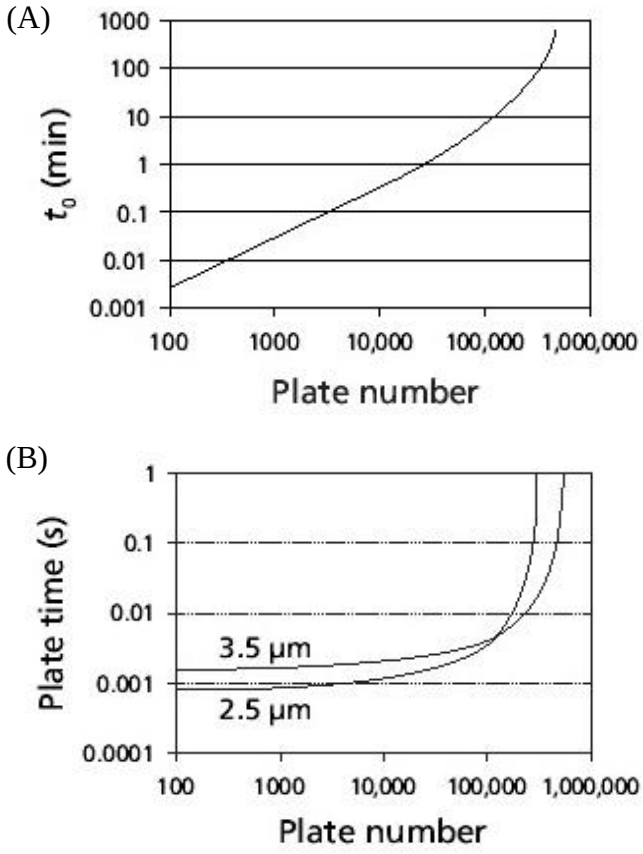


Figure 1.11. Kinetic plot of column dead time (A) and plate time (B) versus plate number [39]. Examples of a plot of column dead time versus plate number plots for 2.5 µm particles (A) and a Poppe plot of plate time versus plate number for 2.5 and 3.5 µm particles (B).

However, the Poppe plot does not consider a link between the back-pressure required for operating a column and the column dead time, making it impossible to compare particles of different porosities. Desmet *et al*. further expanded upon the Poppe plot, using separation impedance (E) as a technique for evaluating column performance, originally proposed by Bristow and Knox [41], to generate a host of kinetic plots. The t_0 plot suffers from a condensed y-axis, making it difficult to read easily. To enlarge the view of the y-axis, t_0 can be divided by N^2, giving a plot of t_0/N^2 versus N, as shown in Figure 1.12. The Desmet team also inverted the plate number (N) axis so that the plot resembles the familiar van Deemter curve, with higher mobile phase velocities and lower plate counts to the right [42].

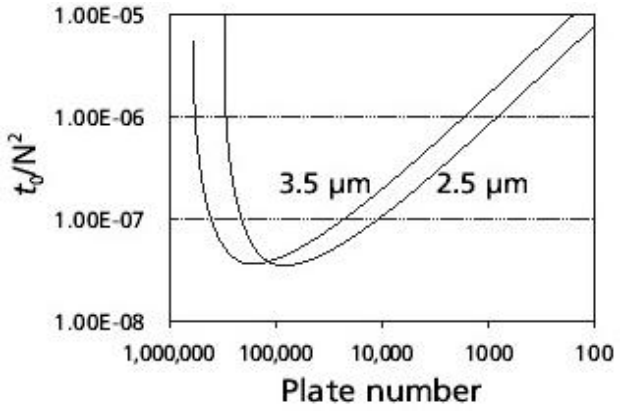


Figure 1.12. Kinetic plot created by Desmet illustrating the comparison of 2.5 and 3.5 µm particles [39].

1.3 Modes of HPLC

Under the umbrella of liquid chromatography, there are various different modes of separation. Depending on the type of sample or analyte, various stationary and mobile phases can be employed to alter the mode of interaction and hence the separation of the particular analytes in question. The major modes of HPLC are normal-phase chromatography (NPC), reversed-phase liquid chromatography (RPLC), ion-exchange (IEC), size-exclusion (SEC) and more recently hydrophilic interaction liquid chromatography (HILIC). Additionally, mixed mode phases such as RP-HILIC and RP-IEC have been [43]. The use of supercritical fluid chromatography (SFC), a form of NPC which utilises supercritical carbon dioxide as the mobile phase, is frequently used for chiral separations [44]. However, this current work focuses on separations by RPLC and HILIC which will be discussed below.

1.3.1 Normal-phase chromatography

The first mode of HPLC to be developed was normal-phase chromatography (NPC), which incorporates a polar stationary phase, typically silica, and non-polar solvents as the mobile phase, for example hexane. It relies on the interaction of polar functional groups of the analytes with polar functional groups on the surface packing. This interaction is mediated by the interaction of the mobile phase with the polar functional groups on the surface of the packing. The mechanisms of interaction are mostly dipole-dipole and hydrogen-bonding. Classical stationary phases are silica or alumina, capable of these interactions by the presence of Si-OH and Al-OH groups. However, there are several bonded phases available, including aminopropyl, cyanopropyl, diol and nitrophenyl. NPC offers a very powerful separation tool because of the wide range of solvents available to fine-tune selectivity, and has been shown to be particularly useful for the separation of non-polar compounds and isomers. However, the disadvantages associated with the technique have seen it fall out of favour, for example the complexities involved, lengthy re-equilibration and reproducibility problems. These are largely due to the sensitivity of the technique to the presence of small concentrations of polar contaminants in the mobile phase. However, these problems can be controlled to provide a superior technique because of the low viscosity solvents employed, but these tend not be environmentally friendly or compatible with MS.

1.3.2 Reversed-phase chromatography

Reversed-phase liquid chromatography (RPLC) is now the most commonly used form of LC, because of its ease of use, reproducibility and broad applicability, and is responsible for between 70-80 % of HPLC separations [35, 45]. In RPLC, separation

is based on an analyte's partition coefficient between a non-polar stationary phase and a polar mobile phase. The stationary phase is commonly based on a silica support, to which hydrophobic ligands, such as octadecyl (C_{18}) groups, are permanently bonded. The polar mobile phase is usually a mixture of a water-miscible organic solvent, typically acetonitrile or methanol, with water or an aqueous buffer. Separation is primarily attributed to partitioning of the lipophilic portion of the analyte into the stationary phase. Based upon hydrophobic interactions, this mechanism results in analytes eluting in order of decreasing polarity; non-polar analytes being more strongly retained due to interactions with the hydrophobic groups of the stationary phase. The extent to which a compound is retained depends primarily upon its lipophilicity and is affected not only by the stationary phase, but also the nature of the mobile phase. The more polar the mobile phase, the quicker a component will elute from a reversed-phase column.

1.3.2.1 Inorganic stationary phase materials

Silica particles commonly act as the base of the stationary phase, composed of silicon atoms joined together by siloxane bonds (≡ Si – O – Si ≡). Bonded phases are formed by covalently attaching the desired organic moiety (e.g. C_8, C_{18}) with silanol groups on the silica surface. The nature of the organic moiety determines the chemistry of the stationary phase and hence the type of interaction that will take place between the solute and the surface. However, steric hindrance prevents these often bulky ligands from reacting completely with all silanols, resulting in only two-thirds of silanol groups being occupied after bonding. Unreacted silanol groups are undesirable and may act as adsorption sites, causing peak tailing and excessive retention [46, 47]. Therefore, a second reaction step, known as end-capping, is

performed to block such sites with small, highly reactive silanes such as trimethylsilanyl groups by treatment with trimethylchlorosilane Figure 1.13. End-capping results in an additional third of active silanol groups being covered with the smaller silanes and, although effective in reducing silanol activity, steric hindrance prevents complete blockage of residual silanols with as many as 50 % of the original silanol groups remaining unreacted on a typical RP column. Since residual silanols are acidic, they can therefore interact with protonated bases via an ion-exchange mechanism causing peak tailing [48]. The manufacture of pure Type B silicas have a far lower metal content compared with older, less purified Type A silica supports, and therefore have reduced silanol acidity [48].

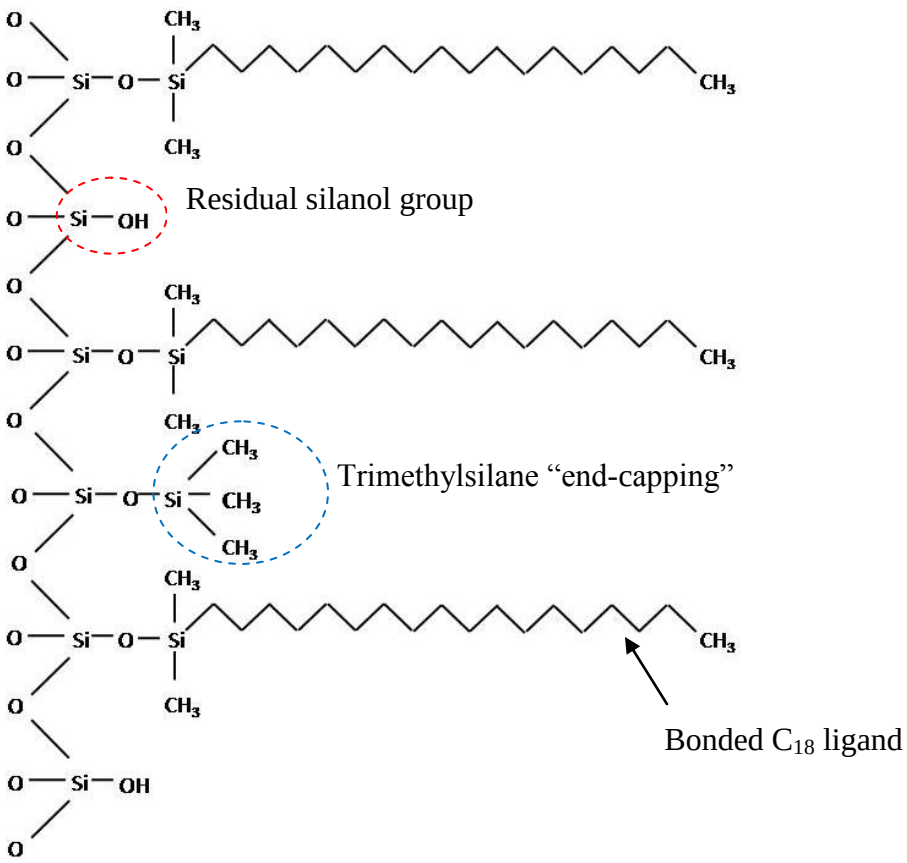


Figure 1.13. Bonded silica (C_{18}) illustrating residual silanol groups and end-capping.

1.3.2.2 Porous graphitic carbon

Porous graphitic carbon (PGC), or Hypercarb as it is known commercially, is composed of flat sheets of hexagonally arranged carbon atoms. Hypercarb is different from traditional silica in its structure and retention properties, and is stable over the entire pH range. This stationary phase has previously shown good retention and separation of highly polar species [49], with a combination of two interaction mechanisms contributing to retention; adsorption and charge-induced interactions [50]. Dispersive interactions between the analyte and mobile phase and the analyte and graphite surface contribute to the strong retention, increasing as the hydrophobicity of the analyte increases. The polarisability of the surface of graphite explains the retention of polar analytes, resulting in a charge-induced dipole.

1.3.2.3 Hybrid stationary phase materials

The inorganic (silicon) and polymeric (carbon) stationary phase supports described above each have distinct advantages and disadvantages, as summarised in Table 1.1. Organic polymers offer advantages in improved peak shapes for basic analytes and high pH stability, but are characteristically less efficient and not as mechanically strong compared with silica-based supports [51]. Silica-based stationary phase materials have been the most commonly employed in RPLC separations, owing to their excellent efficiency, rigidity and stability [52]. However these stationary phase materials often constitute disadvantages for the analysis of basic compounds, resulting in broad, tailing peaks. The nature of silica-based sorbants being sensitive to pH restricts operation to between pH 2 and 7. Below pH 2, such adsorbents are susceptible to hydrolysis [53], whereas above pH 8, hydroxyl groups lead to

dissolution of the phase, causing loss of column efficiency, increased back-pressure and eventual collapse of the packing material [54].

Table 1.1. Summary of inorganic and polymeric packing materials.

	Advantages	**Disadvantages**
Inorganic (silicon)	• Mechanically strong • High efficiency • Predictable retention • Wide particle size range	• Limited pH range • Tailing peaks for bases • Chemically unstable
Polymer (Carbon)	• Wide pH range • No ionic interactions • Chemically stable	• Mechanically unstable • Low efficiency • Unpredictable retention • Often limited particle size range

The preferred stationary phase material would, therefore, combine the advantages of silica gel and polymer based materials. Over recent years, research has focused on synthesising alternative chromatographic media capable of operating at a high pH without sacrificing the excellent performance benefits of silica-based materials. Attempts to reduce interactions between residual silanol groups and ionised basic compounds, either by changing the internal or surface structure of packing materials or by shielding them from the analytes, have resulted in the development of novel modified silica-based supports. Such new generation materials include hybrid organic-inorganic phases, which combine both inorganic and organic elements, therefore sharing the benefits of both silica and organic polymers. The resulting materials have the mechanical strength and high efficiency of inorganic silica materials with the wide pH range and lack of tailing peaks provided by organic materials. These hybrid inorganic-organic phases include the ethylene bridged hybrid

material (BEH Technology™) from Waters, which incorporates ethyl groups throughout the phase as well as on the particle surface [55] (Figure 1.14). During synthesis, one in three silanols is replaced with an ethyl group giving the particles a bridged ethylsiloxane/silica hybrid structure and reduced silanol concentration. Fewer silanol groups mean fewer potential sites for interaction with ionised basic compounds, while the presence of ethyl groups also reduce the acidity of surface silanols by increasing their pK_a (to > 8) compared to that typical of silica (pK_a 3.5-6.8), resulting in further suppression of silanol activity [56]. Presence of the organic polymer slows the rate of dissolution since there are fewer underivatised silanols on the silica surface, which limits potential sites of attack by hydroxyl groups. Hybrid particle phases therefore demonstrate exceptional lifetimes at high pH, which facilitates the manipulation of mobile phase pH for the separation of basic compounds. In addition, the high degree of cross-linking offers excellent mechanical strength providing BEH particles with the capacity to withstand the high pressures experienced in UPLC®.

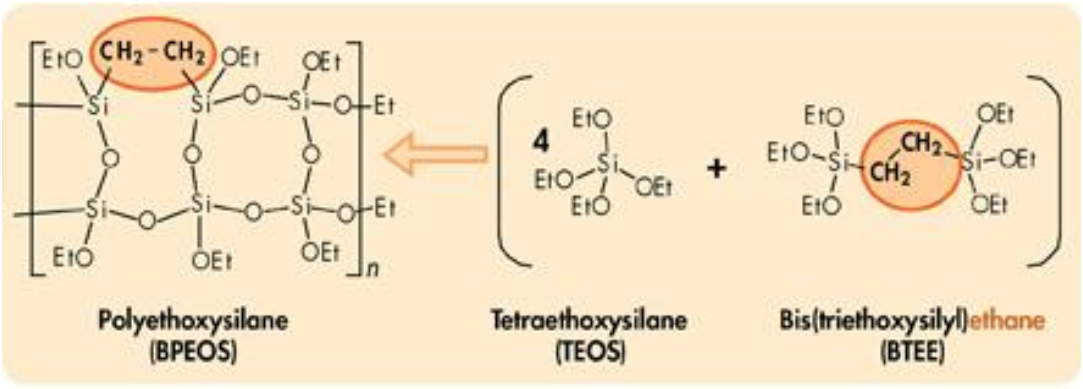


Figure 1.14. Bridged ethyl hybrid technology (BEH Technology™, Waters Corporation) [57].

1.3.3 Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a variant of NPC, so called by Alpert *et al.* in 1990 to distinguish the two techniques [58]. Although HILIC has been practised for a long time, it has recently emerged as an important technique for the analysis of very polar compounds such as carbohydrates and polar peptides. Inverse to RPLC, in HILIC, retention increases as analyte polarity increases and decreases as mobile phase polarity decreases. The stationary phases are polar and used with a highly organic mobile phase containing a small proportion of water or buffer, as illustrated in Figure 1.15. As for RPLC, the gain in popularity of HILIC has resulted in a variety of bonded phases available in addition to the traditional underivatised silica or hybrid materials, including amide, amino, diol and zwitterionic phases offering different properties for a range of applications. HILIC mobile phases generally contain more than 70 % acetonitrile and, for the formation of a hydrophilic layer on the surface of the packing, at least 3 % of the mobile phase should contain a polar solvent [59]. Additionally, HILIC mobile phases typically contain an additive or buffer to aid retention and peak symmetry.

Alpert *et al.* proposed that the main mode of interaction was due to partitioning of solutes from the bulk organic mobile phase with the partially-immobilised aqueous layer at the stationary phase. The mechanisms behind this technique have been extensively reviewed and continue to be debated with several contributing modes of interaction now considered to influence retention, including hydrogen bonding, dipole-dipole interactions and electrostatic interactions [60]. Ion-exchange mechanisms are commonly encountered, since many of the stationary phases

employed have ion-exchange properties. However, this can be exploited to manipulate the selectivity of a separation.

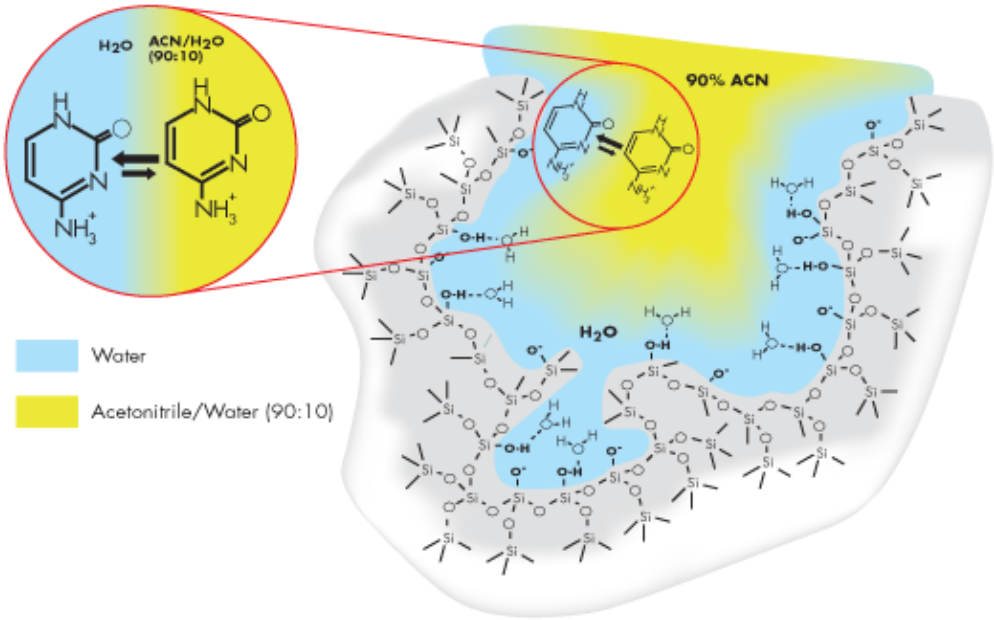


Figure 1.15. Illustration of hydrophilic interaction liquid chromatography (HILIC) [61].

1.3.4 HPLC Method Development

The resolution (R_s) between two solutes can be affected by both thermodynamic factors (retention, k, and selectivity, α) and kinetic factors (peak width and column efficiency, N). Therefore the Purnell, or resolution equation, which relates to efficiency (N), selectivity factor (α) and the retention factors of the two solutes in a separation, is useful for improving the conditions that directly affect resolution:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{k_2}{k_2+1}\right)\left(\frac{\alpha-1}{\alpha}\right)$$

where R_s is the resolution, N is the number of theoretical plates, k_2 is the retention factor of the second peak of the two solutes and α is the selectivity factor.

To obtain the best resolution, all three terms must be maximised. For example, N can be increased by reducing particle size or increasing column length. The retention factor can be controlled by varying the mobile phase composition (% B). Ideally, k will be neither too small nor too large, with a typical goal of $2 \leq k \leq 10$, providing sufficient retention to avoid overlap with matrix interferences, but still generating narrow and tall enough peaks for sensitive detection. However, the selectivity factor, α, is the most powerful term that can be varied to maximise resolution. Selectivity can be manipulated by changing the mobile phase composition, type of organic solvent used, column chemistry or column temperature and, in the case of ionisable compounds, mobile phase pH, buffer concentration or ion-pairing reagent concentration.

1.3.4.1 Stationary phase selectivity

With the wide complement of stationary phases now available, column selectivity offers a major parameter in method development and optimisation. Considering RPLC, there are many different bonded chemistries and brands of column packing materials, each differing in their hydrophobicity, silanophilic activity, shape selectivity, polar selectivity and metal content. Even stationary phases of the same chemistry vary from manufacturer to manufacturer, depending on the type of support used, degree of carbon loading and end-capping process.

An additional advance in the stationary phase development is the incorporation of a polar functional group into the ligand, close to the surface of the silica, resulting in the polar-embedded phase [62, 63]. Such bonded ligands are effective in "shielding" silanol groups, preventing interaction with basic analytes through steric hindrance.

An additional benefit brought about by such chemistry is the improved wettability in highly aqueous mobile phases. With traditional hydrophobic ligands, it is necessary to maintain a degree of organic solvent in the mobile phase (> 5 %) to prevent phase de-wetting. Hence, such polar-embedded phases are advantageous for the analysis of highly polar analytes, which necessitate fully aqueous mobile phases to obtain sufficient retention.

1.3.4.2 Mobile phase selectivity

Changes in solvent strength, either the concentration or type of organic component used, provides a convenient means of altering selectivity. In RPLC, acetonitrile or methanol is usually a first choice, with acetonitrile presenting a stronger solvent and therefore typically generating smaller *k* values and less resolving power. For the separation of very polar analytes by RPLC, sufficient *k* can be achieved if minimal organic solvent is used. This historically resulted in phase de-wetting, due to expulsion of mobile phase from the pores of particles. However, the polar embedded phases now available are able to tolerate operation in fully aqueous mobile phases required for the retention of very polar species.

The ability to modify mobile phase pH provides the chromatographer with an additional tool in order to achieve suitable retention and peak shape of basic analytes. The pH restrictions of traditional silica materials render basic analytes protonated under these conditions, which is detrimental to chromatography. However, the stability of newer stationary phase materials over a wide pH range allows the pH of the mobile phase to be increased in order to suppress analyte protonation, which inherently reduces secondary interactions between the positive

analyte charge and the negative silanol groups responsible for poor peak shape. In addition, retention is enhanced, since polarity is reduced as analyte ionisation is suppressed, permitting hydrophobic interactions to dominate the mechanisms of retention. This was previously not possible with the limited pH range of the stationary phase of between pH 2-7, since a pH of two units above the pK_a of the analyte is required to completely suppress basic analyte ionisation.

Ion-pair reagents provide another opportunity to affect selectivity. The use of trifluoroacetic acid (TFA) and triethylamine (TEA) have been show to improve chromatographic separations, although this option has become less popular because of the incompatibility with MS ionisation and detection.

1.3.4.3 Temperature selectivity

Column temperature is an important variable which can have a significant influence on selectivity, causing retention changes according to the van't Hoff equation, expressed as:

$$\log(k) = -\frac{\Delta H_0}{2.3RT} - \frac{\Delta S_0}{2.3R} - \log \phi$$

where ΔH_0 and ΔS_0 are the enthalpy and entropy of solute transfer, respectively, from the mobile phase to the stationary phase, T is the absolute temperature, R is the universal gas constant and $\emptyset$ is the phase ratio of the column.

As temperature increases, retention often decreases, resulting in poorer separation, while peak heights increase, providing better sensitivity. However, in certain cases an increase in temperature can lead to an increase in retention, and changes in the order of elution can therefore be observed through varying column temperature [64-66]. Separations are typically performed between ambient and 60 °C, although with the advent of more stable stationary phases, operation at temperatures in excess of 100 °C are now possible [67, 68]. In addition, increased column temperature has the added kinetic benefits of lowering column back-pressure, since mobile phase viscosity is reduced. This enables the use of longer column formats if a higher plate number is required for improved resolution, or the use of faster flow rates to reduce analysis time. However, while increased column temperature offers these advantages, certain disadvantages should also be considered such as analyte stability and stationary phase hydrolysis.

1.4 Mass Spectrometry

Mass spectrometry (MS) is an unequalled analytical method for sensitive analyte detection, identification and quantification. Molecules are ionised to generate charged molecules, or charged molecular fragments, which are measured according to their mass-to-charge (*m/z*) ratios in a high vacuum. This enables the determination of isotopic molar mass, elemental composition and elucidation of chemical structure.

The ions produced must be in the gas phase so they can be manipulated by the application of either electric or magnetic fields to enable separation. A conceptual illustration of a mass spectrometer is given in Figure 1.16, comprising three modules: an ion source where ions that exist in solution are moved into the gas

phase, a mass analyser which separates ions by their *m/z* ratios and a detector which records the current generated when a charged particle hits the detector to obtain a mass spectrum.

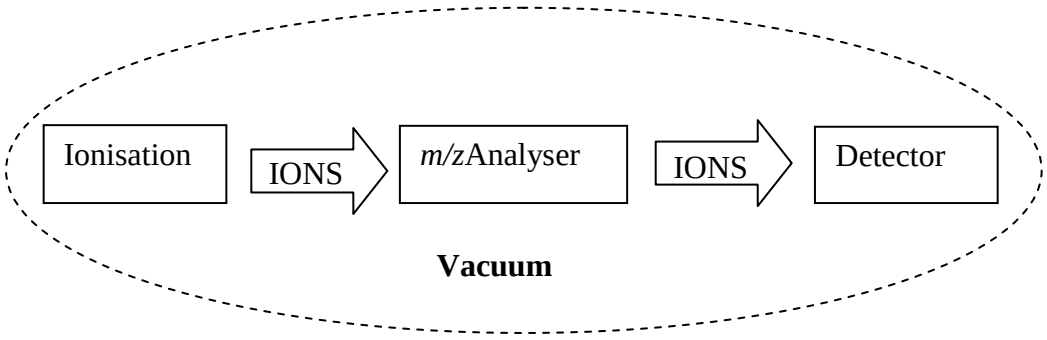


Figure 1.16. Conceptual illustration of the mass spectrometer.

1.4.1 Ionisation

The initial connection of HPLC with MS was a huge challenge, convoluted by thermally labile and/or non-volatile analytes. With sample dissolved in a solvent, such as that eluting from a LC platform, an interface is required to transform the mobile phase from the liquid to the gas phase. The development of an LC-MS interface has resulted in a collection of ionisation modes conducted at atmospheric pressure (API), including electrospray ionisation (ESI) [69], atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI). The development of API techniques, in particular ESI, enabled LC-MS to finally gain popularity as one of the most powerful analytical platforms. The ionisation techniques employed in this thesis will be discussed below.

1.4.1.1 Electrospray Ionisation

Electrospray ionisation (ESI) was a pioneering advancement in the hyphenation of LC with MS and remains the most commonly used ionisation technique in LC-MS due to its wide applicability. This soft form of ionisation produces minimal fragmentation and is used for ionic, neutral and polar compounds. As illustrated in Figure 1.17, the LC eluent passes, at atmospheric pressure, through a capillary tube to which a large voltage is applied in order to produce a spray of fine droplets. The potential difference applied, typically several thousand volts, generates an electric field between the capillary and the counter electrode (cone). Depending on the analytes of interest, this voltage may be positive (positive ionisation) or negative (negative ionisation). This potential serves to provide charge separation of the ions at the surface of the liquid at the tip of the capillary to form a "Taylor cone". Through gentle desolvation by a heated drying gas, typically nitrogen, droplets continue to diminish in size with the evaporation of solvent. The charge accumulates at the surface of the droplet, since ions (which are involatile) are retained and the concentration of the analyte increases. There are then two debated mechanisms which occur, referred to as the charge residue model (CRM) [70] and the ion evaporation model (IEM) [71, 72]. A decrease in droplet size increases the repulsive forces between the excess charge in the droplet, promoting "Coulombic explosion". In the CRM, this process continues until the point where the charge repulsion on the surface of the droplet overcomes the surface tension, known as the Rayleigh limit. This mechanism eventually results in single ionised molecules and assumes that the increased charge density due to solvent evaporation causes large droplets to divide into smaller and smaller droplets. The second proposed IEM mechanism assumes that the increased charge density that results from solvent evaporation eventually

causes Coulombic repulsion to overcome the liquid's surface tension, resulting in a release of ions from droplet surfaces.

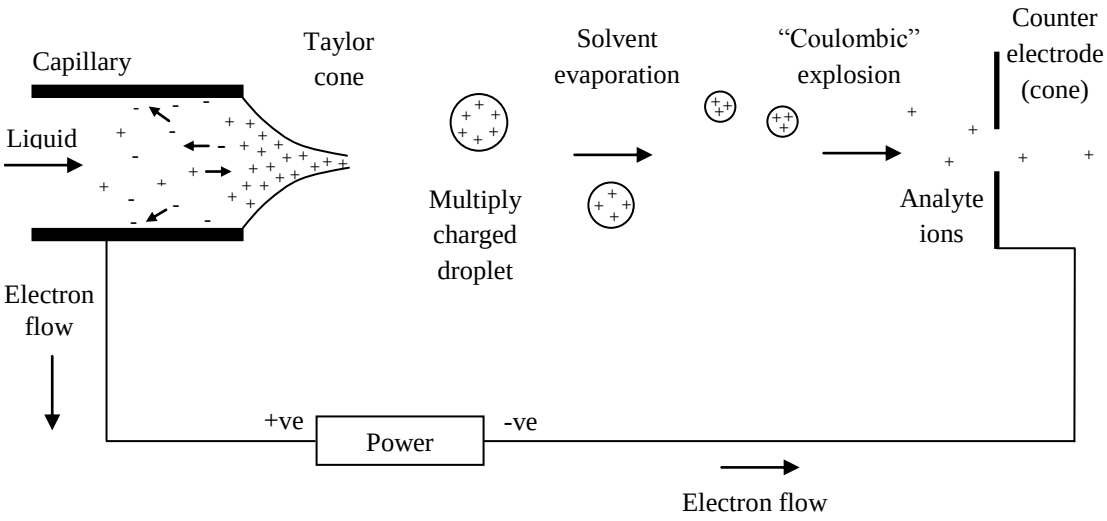


Figure 1.17. Illustration of positive electrospray ionisation (ESI).

1.4.1.2 Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (APCI) is another form of soft ionisation, mainly applied to the analysis of polar and relatively non-polar compounds. In APCI, the analyte in solution is introduced into a pneumatic nebuliser and desolvated in a heated quartz tube before interacting with a corona discharge to create ions. Ionisation occurs in the gas phase, as described in Figure 1.18, with the corona discharge providing low-energy electrons to initiate the gas-phase reactions. These low-energy electrons ionise a reagent gas from solvent molecules (e.g. N_2, O_2, H_2O, etc) that, through a complex series of ion/molecule reactions, efficiently produce positive and negative ions of the analyte. Secondary reactant gas ions (e.g. H_3O^+) undergo repeated collisions with the analyte, resulting in the formation of analyte ions. The frequency of collisions results in a high ionisation efficiency and

thermalisation of the analyte ions. APCI results in spectra of predominantly molecular species and adduct ions with very little fragmentation.

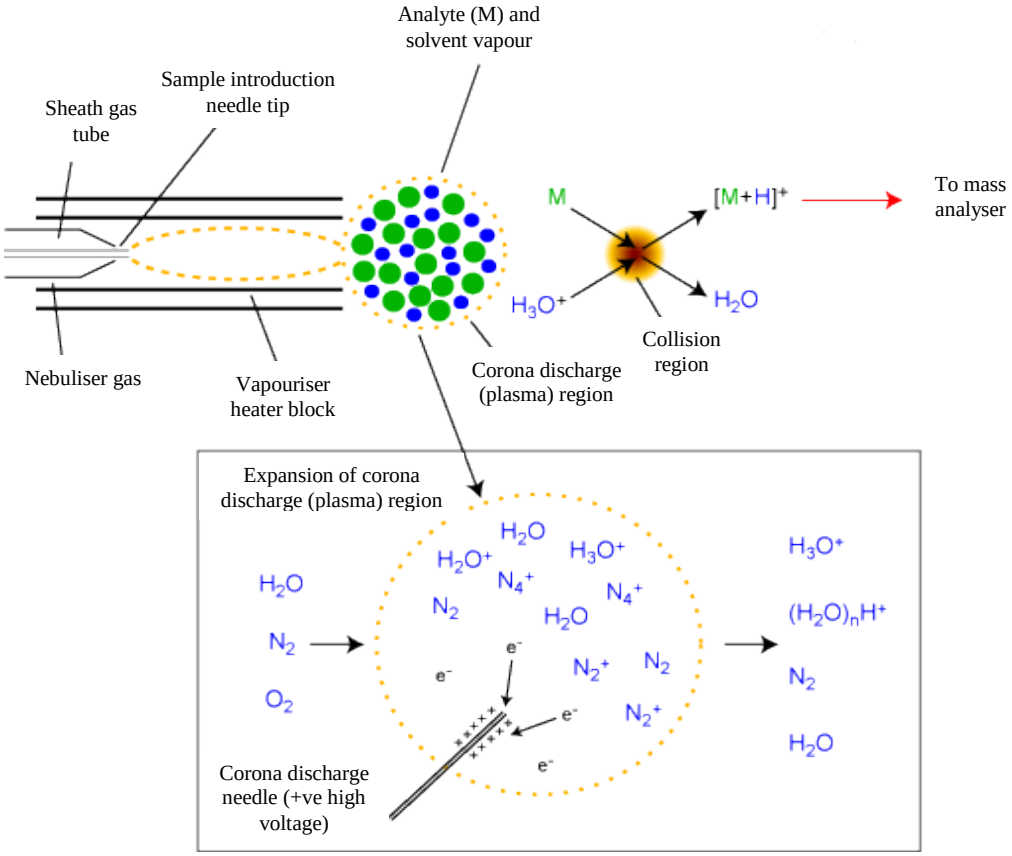


Figure 1.18. Illustration of APCI [73].
(Obtained from http://www.bris.ac.uk/nerclsmsf/images/apci2.gif)

1.4.2 Mass Analysers

Once formed, ions are accelerated and focused into a mass analyser where they are separated according to their *m/z* ratio. Several types of mass analysers have been developed which can be interfaced with LC. However, in the current study, quadrupole transmission and time-of-flight (TOF), including tandem and hybrid instruments, have been employed and will be discussed here.

1.4.2.1 Quadrupole analysers

The quadrupole mass analyser consists of four parallel rods to which two varying electrostatic fields are applied at right angles to each other; one direct current (DC) and one radiofrequency (RF) (Figure 1.19). Ions produced in the source of the instrument are focused and passed through the centre of the four rods. Ions are able to transverse the field-free region along the centre of the axis of the rods where the oscillations amongst the poles result in ion trajectories dependent on the *m/z* of the ions. Ions with a particular *m/z* have a stable trajectory and pass to the detector, whereas those with an unstable trajectory will hit the quadrupoles and not be detected. The mass range and resolution of the instrument is dependent on the length and diameter of the rods. The full mass range is normally achieved by scanning the RF voltage and DC voltage together so that the RF/DC ratio is constant.

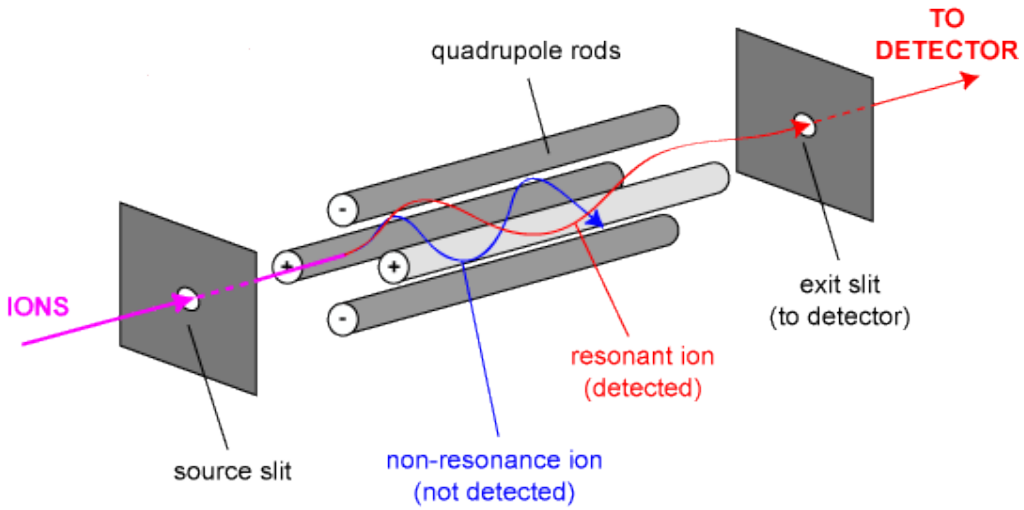


Figure 1.19. Schematic representation of a quadrupole mass filter [74].
(Obtained from http://www.chm.bris.ac.uk/ms/theory/quad-massspec.html)

1.4.2.2 Tandem mass spectrometry

Quadrupoles are often combined in sequence to provide a tandem process which enables fragmentation studies to gain additional structural information. The most

common configuration is three quadrupoles coupled in series, referred to as tandem or triple quadrupole (QQQ) mass spectrometry, which enables basic ion fragmentation (tandem MS/MS). Figure 1.20 illustrates a triple quadrupole analyser, where the first quadrupole (Q1) selects the precursor ions, which are then transferred into the second quadrupole (Q2), an ion guide where collision with an inert gas, typically argon or nitrogen, promotes analyte fragmentation. The fragments, or product ions, are then separated by the third quadrupole (Q3) to give a characteristic mass spectrum.

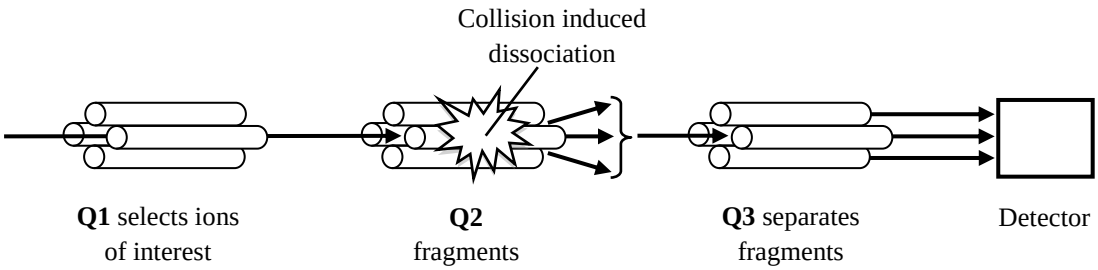


Figure 1.20. A schematic representation of a triple quadrupole mass analyser.

The triple quadrupole mass spectrometer is still the most commonly used detection technique for quantitative analysis of small drug molecules owing to its unrivaled sensitivity and selectivity in addition to the high ion throughput obtainable.

1.4.2.3 Time-of-flight analysers

Time-of-flight (TOF) mass spectrometry is one of the earliest type of instruments but was limited because of the speed of early electronics and ionisation techniques. TOF mass analysers rely on separation of ions, after their initial acceleration through an electric field, according to their velocities as they transverse through a field-free region called the flight tube. Acceleration by an electric field of a known strength

results in ions of the same charge having the same kinetic energy. The time taken for ions to move through the flight tube of a known distance to the detector determines their *m/z* ratios. This depends on their velocity, with larger ions reaching lower speeds and taking longer to reach the detector. The advantage of a TOF is the ability to detect all ions, compared with other mass analysers that filter selected ions. Since the operation of TOF MS relies on the drift of ions, the length of the flight tube is of major importance for resolution, with the longer the flight, the greater the resolution obtained. Analytical use of the technique has historically been limited by the length of the flight tube required to achieve adequate mass resolution. However, the use of reflectrons now provides increased resolving power, since the effective length of the flight tube can be doubled when ions are reflected at the end of the flight tube. Latterly, TOF-MS has gained further popularity because of the high resolving power that could be achieved through reflectron technology and accurate mass capabilities, and advances in superior ion transmission have resulted in improved detection limits. The rapid spectral acquisition rates of the TOF are another important feature, with the ability to produce several thousands of spectra per second. TOF instruments have been traditionally used for the analysis of relatively high molecular mass compounds, such as proteins, but with recent innovation there has recent interest in the application of these instruments for small molecule analysis.

1.4.2.4 Quadrupole time-of-flight analysers

Several mass spectrometers combine different types of analysers, referred to as hybrid instruments. Tandem mass spectrometry is feasible with a TOF instrument, typically combining a quadrupole and collision cell between the source and the TOF analyser (Figure 1.21). Such hybrid quadrupole TOF (QTOF) mass analysers have

become increasingly popular over recent years, providing the attractive combination of the ability to select ions for MS/MS fragmentation with high resolution and accurate mass measurements. Traditionally, these instruments have mainly been used for qualitative work due to their inherent limited dynamic range, although improvements in gating mechanisms have recently improved this. The tandem QTOF mass spectrometer has been used extensively for proteomics but has also found many uses in the area of small molecule analytical chemistry [75-77].

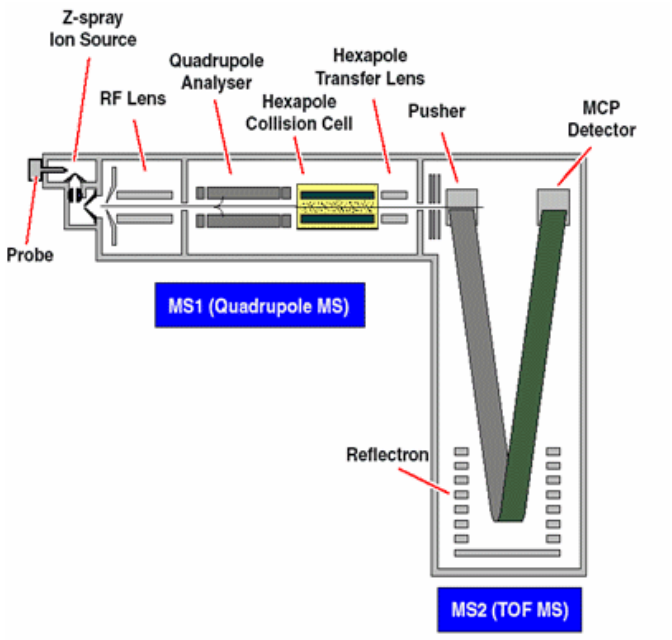


Figure 1.21. Illustration of the QTOF instrument [78].
(Obtained from http://www.whri.qmul.ac.uk/PAU/MSunit_QTOF.htm)

1.4.3 Considerations for LC-MS hyphenation

Stable MS spray and analyte ionisation can be affected by several parameters; the instrument hardware or design, analyte characteristics and the LC conditions employed before the sample is introduced into the source. Mobile phase composition and flow rate are important considerations when coupling LC with MS. The use of mobile phase additives and buffers can not only dramatically affect the

chromatography, but also the MS response. Although the mechanisms surrounding ESI are still not fully explained, there are several reports on how mobile phase composition and components of the sample matrix can impact on the results of MS analysis.

1.4.3.1 Mobile phase composition

The influence of the solvent composition has a significant effect on ESI signal and is a feature of the separation developed to introduce the sample to the MS. Increasing the organic content in the mobile phase results in increased ionisation due to changing the solvent characteristics such as viscosity, conductivity and surface tension [79]. Many of the buffers or ion-pairing agents commonly employed to improve chromatographic separation are not compatible with MS detection. Even the use of volatile buffers can introduce more surface-active electrolytes, which will compete with the target compound and suppress ionisation, and the pH as well as the type of additive affects the amount and type of ions formed [79-82]. Ion-pairing agents, such as TFA and TEA, have been shown to have a negative impact on ionisation, where the ion pair may not fully dissociate and therefore appear as a neutral species, resulting in ion suppression [83-85].

1.4.3.2 Ion Suppression

An important consideration for a selective method is the assessment and quantification of sample matrix effects, where co-eluting endogenous species have the potential to suppress, or enhance, ionisation. This is of particular importance in bioanalysis, where complex matrices, such as urine, plasma and serum, are

frequently analysed. While ion suppression has been observed with both APCI and ESI, APCI has been shown to be less susceptible to matrix effects [86].

The investigation of how matrix effects are reflected in MS response is paramount when developing LC-MS methodology, especially if quantification is required. Once identified, matrix effects can be minimised, if not eliminated, through appropriate sample pre-treatment or chromatographic optimisation. The use of selective sample preparation, including solid phase extraction (SPE) and liquid-liquid extraction (LLE), is often an important step in further minimising matrix effects that could occur with ESI-MS. However, where sensitivity is not of great importance, LC is compatible with a simple dilution and injection approach. There are several reports illustrating the success of direct sample dilution, centrifugation and injection for fast, high-throughput analysis without any negative impact on the MS signal, although much attention must be paid to ensure there are minimal matrix effects if this approach is taken [31, 87]. Since UHPLC offers enhanced resolution, the risk of co-elution is reduced, which in turn results in a lower risk of ion suppression, improving MS sensitivity and reliability. Van de Steene *et al.* have compared the degree of matrix effects when using HPLC-MS/MS with UPLC-MS/MS for nine pharmaceuticals, concluding that with UHPLC matrix effects were either minor or totally eliminated [88].

1.5 Aims

The purpose of this thesis was to investigate novel approaches to chromatographic separations using sub-2 µm stationary phases with the latest UHPLC technology, enabling fast, sensitive and robust drug analysis. There was a particular focus on the research of chromatographic techniques interfaced with MS, in order to establish the

application of UHPLC-MS to improve existing methods of bioanalysis which are mostly based on GC-MS. Examples are taken from doping control, where the challenges associated with analysing hydrophilic, basic analytes by RPLC, while maintaining MS operation, are addressed, and methodology is developed for the reliable and robust quantification of such analytes. Alternative approaches are studied based on advances in stationary phase materials, such as hybrid supports and HILIC, and MS technology, including the shift from tandem MS/MS to the use of high resolution accurate mass spectrometry (HRAMS) for quantification in bioanalysis.

CHAPTER 2

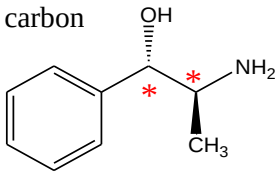
EFFECTS OF MOBILE PHASE PH ON THE ANALYSIS OF BASIC COMPOUNDS BY REVERSED-PHASE HPLC

2.1 Introduction

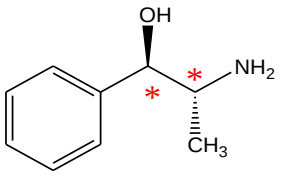
2.1.1 Separation of Basic Compounds

High performance liquid chromatography (HPLC) continues to be dominated by reversed-phase applications. However, the analysis of basic compounds remains a major challenge for reversed-phase liquid chromatography (RPLC), a particular problem since the majority of pharmaceutical compounds comprise ionic, nitrogen-containing functional groups. The ephedrines (norephedrine (cathine), norpseudoephedrine (phenylepropanolamine, PPA), ephedrine, pseudoephedrine and methylephedrine) are examples of such basic compounds which are difficult to separate by RPLC, the structures and pK_a values of which are shown inFigure 2.1. Frequently found in pharmaceutical preparations and nutritional supplements, these analytes have stimulant effects and for this reason their use is restricted in human sport [89]. Administration of these substances is controlled in competition, with the World Anti-Doping Agency (WADA) prohibiting their use above threshold concentrations [90]. Due to the identical elemental composition of the diastereoisomeric pairs (PPA-cathine and ephedrine-pseudoephedrine), they share virtually identical mass spectra and therefore require chromatographic separation for unambiguous identification and accurate quantification. Previously, this separation has been performed by HPLC-UV [91] or GC-MS, which relies upon a sample pre-concentration liquid-liquid extraction followed by complex derivatisation in order to distinguish PPA from cathine and ephedrine from pseudoephedrine [92]. However, as previously discussed, LC-MS has assumed an important role in bioanalysis over the last 15 years, having advantages in simple sample preparation, faster analysis times and increased sensitivity [31, 32, 93-95].

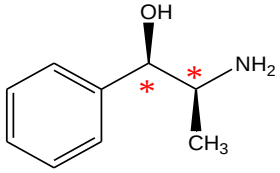
* = chiral carbon



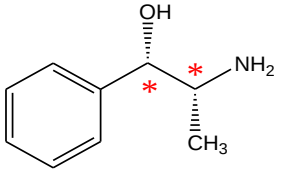
(1S,2S)-norpseudoephedrine (cathine), pK_a 9.4
(1S,2S)-2-amino-1-phenylpropan-1-ol



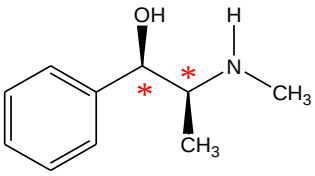
(1R,2R)-norpseudoephedrine (cathine), pK_a 9.4
(1R,2R)-2-amino-1-phenylpropan-1-ol



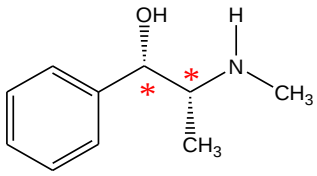
(1R,2S)-norephedrine (phenylpropanolamine), pK_a 9.4
(1R,2S)-2-amino-1-phenylpropan-1-ol



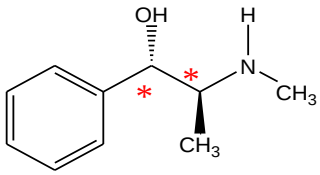
(1S,2R)-norephedrine (phenylpropanolamine), pK_a 9.4
(1S,2R)-2-amino-1-phenylpropan-1-ol



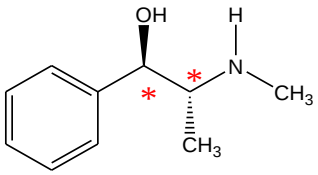
(1R,2S)-ephedrine, pK_a 9.6
(1R,2S)-2-methylamino-1-phenylpropan-1-ol



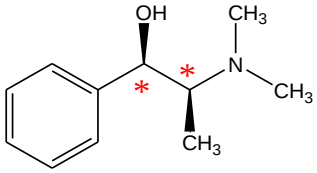
(1S,2R)-ephedrine, pK_a 9.6
(1S,2R)-2-methylamino-1-phenylpropan-1-ol



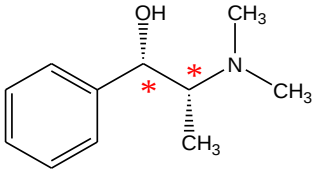
(1S,2S)-pseudoephedrine, pK_a 9.8
(1S,2S)-2-methylamino-1-phenylpropan-1-ol



(1R,2R)-pseudoephedrine, pK_a 9.8
(1R,2R)-2-methylamino-1-phenylpropan-1-ol



(1R,2S)-methylephedrine, pK_a 9.3
(1R,2S)-2-(dimethylamino)-1-phenylpropan-1-ol



(1S,2R)-methylephedrine, pK_a 9.3
(1S,2R)-2-(dimethylamino)-1-phenylpropan-1-ol

Figure 2.1. Chemical structures showing stereoisomerism, IUPAC nomenclature and pK_a values of the ephedrine compounds considered in this study.

Previous alternative approaches to overcoming the inherent challenges associated with analysing basic compounds by RPLC include the use of various different stationary phase chemistries and mobile phase additives. As is the case with the ephedrines, the analysis of ionisable compounds which are also hydrophilic is even more problematic. With typical acidic mobile phases, such cases require very low amounts of organic modifier in order to achieve acceptable retention of protonated bases, or organic additives in order to achieve acceptable peak shapes [24, 96, 97]. The low percentage of organic solvent content (< 5 %) necessary to achieve suitable retention can often cause issues such as phase de-wetting, where the hydrophobic ligands bonded to the silica stationary phase collapse. This may result in retention time shifts and requires regeneration every several injections, thereby limiting sample throughput. Additionally, such highly aqueous conditions are not ideal for MS detection, particularly with electrospray ionisation (ESI), owing to poor desolvation of the eluent, which results in poor analyte ionisation and hence reduced sensitivity. Organic additives, including trifluoroacetic acid (TFA) and triethylamine (TEA), are also frequently employed as counter-ions to interact with the analyte and the stationary phase. Although successful in considerably improving retention, peak shape and separation for ionic and polar analytes, these additives cause ion suppression and have a lingering ion-pair effect that is difficult to remove from a LC-MS system and may even require dedicated instrumentation [98, 99].

An alternative approach to separating basic compounds by RPLC which has recently emerged is to use a high pH mobile phase so as to suppress analyte ionisation, and thus polarity, which allows enhanced retention with reduced peak tailing [48, 100, 101]. Until recently, the use of high pH mobile phases (above pH 8) has been

limited, due to the instability of conventional silica-based stationary phases susceptible to dissolution in high pH conditions [54]. While non-silica phases, such as PGC and polymeric materials, offer stability over a wide pH range and therefore permit the use of high pH mobile phases, their poor efficiency and mechanical stability has limited their use. However, the development of chemically stable phases, such as hybrid inorganic/organic materials, facilitates the use of mobile phases of high pH to suppress the ionisation of basic analytes [51, 102, 103]. The possibility of using mobile phase pH to achieve symmetrical peak shapes and acceptable retention factors for these compounds with an MS compatible system to maintain LC-MS operation is highly desirable. The ability to manipulate mobile phase pH above the analyte pK_a enables suppression of analyte ionisation and permits enhanced retention and resolution in addition to significantly improved peak shapes due to the lack of secondary interactions with ionised silanol groups. In addition, it has previously been reported that in their unionised form, analytes are better able to tolerate the detrimental effects of column overloading [104-107]. Contrary to traditional theory, the use of high pH mobile phases to separate basic compounds in their neutral form has not proven to be detrimental to ionisation in MS, with several reports that such conditions even enhance MS ionisation [108-110].

The present chapter describes the manipulation of mobile phase pH in developing a method to separate phenylpropanolamine (PPA), cathine, ephedrine, pseudoephedrine and methylephedrine. Although capillary electrophoresis might prove a beneficial alternative for the separation of these closely related basic analytes, a method compatible with MS detection was sought after. Therefore, considering the difficulty in coupling CE with MS, a LC approach was adopted. Low

(pH 3), mid (pH 7) and high (pH 10) pH mobile phases are studied to investigate the effects on retention, peak shape, resolution and the sample loading capacity of basic compounds in RPLC. Additional parameters affecting chromatographic performance, including the organic content and temperature, are also investigated with respect to high pH conditions in order to evaluate the effects on retention, resolution, peak shape and column efficiency. In particular, the effects of combining high mobile pH with elevated temperatures are evaluated. Increasing column temperature results in reduced viscosity and back-pressure and increased diffusion coefficients, providing a decrease in resistance to mass transfer [66, 111-113]. These benefits are obvious with van Deemter curves displaying flatter slopes and therefore generating higher optimal linear velocities. Faster flow rates can therefore be realised, leading to faster analysis times without sacrificing efficiency, and the lower back-pressures at higher temperature permit the use of longer or coupled columns in order to generate high plate numbers [7, 114].

The generation of van Deemter curves at low and high pH demonstrates the effect of pH and temperature on the efficiency of separation to obtain the optimal conditions. In addition, kinetic plots are used to evaluate the differences in kinetic performance between low and high pH mobile phase conditions. An optimised HPLC separation of the five ephedrines is then compared with UPLC® technology.

2.3 Experimental

2.3.1 Materials

Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium hydroxide solution (35 % w/w) and ammonium bicarbonate were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99-100 %) was purchased from VWR (Leicestershire, UK). Ammonium acetate and ammonium formate were purchased from Sigma (Poole, UK). Norephedrine (phenylpropanolamine, PPA), norpseudoephedrine (cathine), ephedrine, pseudoephedrine and methylephedrine were purchased as hydrochloride salts from Sigma (Poole, UK). Water was purified by an ultra-pure water system (Millipore, UK).

2.3.2 Solutions

2.3.2.1 Mobile phase

10 mM solutions of ammonium formate, ammonium acetate and ammonium bicarbonate were prepared in purified water. Ammonium formate was adjusted to pH 3 with formic acid and ammonium bicarbonate was adjusted to pH 10 with ammonium hydroxide solution.

For the final method, a stock solution of ammonium bicarbonate buffer was prepared at 25 mM in purified water and adjusted to pH 9.8 with ammonium hydroxide solution (35 % w/w). This buffer was then used for the preparation of the final mobile phase, which consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60 % methanol (B). For the preparation of 1 L of mobile phase A, 400 mL of the stock buffer solution was added

to 600 mL of water in order to achieve a 10 mM buffer solution. For the preparation of mobile phase B, 200 mL of the stock buffer was added to 300 mL of methanol.

2.3.2.2 Samples

Stock solutions were prepared at a concentration of 1 mg/mL for cathine, ephedrine and methylephedrine and 10 mg/mL for pseudoephedrine in methanol and stored at -20 °C. Standard working solutions were prepared by diluting stock solutions with the appropriate mobile phase.

2.3.3 LC conditions

All separations were carried out on an Acquity UPLC® system (Waters, Milford, MA, USA) with an XBridge C_{18} 2.5 µm, 2.1 x 50 mm column or an Acquity BEH C_{18} 1.7 µm, 2.1 x 50 mm column provided with a 0.2 µm in-line filter. Unless otherwise stated, investigations were carried out using 20:80 v/v CH_3OH: aqueous buffer with a flow rate of 400 µL/min and a column temperature of 30 °C.

For the final optimised method, the run time was 6.5 minutes including the re-equilibration time. The mobile phase consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60 % methanol (B). The flow rate was 500 µL/min and column temperature was set at 45 °C. The weak and strong needle wash lines of the Acquity UPLC® system were placed in 90:10 H_2O/CH_3OH 0.2 % formic acid and 10:90 H_2O/CH_3OH 0.2 % formic acid respectively. The injection volume was 2 µL and injections were performed in the full loop mode using a 2 µL sample loop. The gradient conditions started at 16.7 %

B, increasing to 41.7 % over 3.2 minutes and to 91.7 % at 5.2 minutes, returning to 16.7 % for a 1.3 minute re-equilibration.

2.3.4 Detection

An Acquity PDA detector (Waters, Milford, MA, USA) was connected to the outlet of the UPLC®. The detector was equipped with a 500 nL flow cell and solutes were detected at 210 nm with a sample rate of 40 Hz. The chromatograms were processed using Empower software (Waters, Milford, MA, USA).

2.3.5 Construction of van Deemter and kinetic plots

Experimental plate height, *H*, and mobile phase linear velocity, u_0, values were obtained using a 5 cm column at pH 3 and 10 at 30 °C, and for pH 10 at column temperatures of 45 and 60 °C. The test mixture was composed of uracil (void volume marker), PPA, ephedrine and methylephedrine. All experiments were conducted in the isocratic mode using pre-mixed mobile phases. The organic content of the mobile phases was varied between pH 3 and pH 10 in order to maintain similar retention factors, hence 5:95 v/v CH_3OH: ammonium formate and 20:80 v/v CH_3OH:ammonium bicarbonate were used at pH 3 and pH 10, respectively. All injections were made in duplicate and the results averaged. The theoretical plate count (*N*) for each analyte was calculated from the peak widths at half height using Empower™ software (Waters, Milford, MA, USA). The u_0 and *H* values were calculated using the following equations:

$$H = \frac{L}{N}$$

$$u_0 = \frac{L}{t_0}$$

where L is the column length, N is the number of theoretical plates and t_0 is the void time. Van Deemter curves were determined by fitting the H versus u_0 data with a nonlinear curve fitting function in SigmaPlot 12.

The viscosities in the different methanol-buffer mixtures and temperatures were calculated according to the empirical relationship derived from the Chen-Horvath equation [66]:

$$\eta = 10^{\left(-2.429+(714/T)-1.859x_{MeOH}+(912/T)x_{MeOH}+1.859x^2{}_{MeOH}-(968/T)x^2{}_{MeOH}\right)}$$

where x is the fraction of methanol in the mobile phase and T is the temperature in Kelvin.

The calculated viscosities are detailed in Table 1. The diffusion coefficients of the solutes were calculated using an expression derived from the Wilke-Chang equation [115]:

$$D_m = 7.4 \times 10^{-8} \frac{\sqrt{x_{org}\psi_{org}MW_{org} + x_{water}\psi_{water}MW_{water}}T}{\eta {V_A}^{0.6}}$$

where x_{org} and x_{water} are the molar fractions of the organic component and water in the mixtures, respectively, ψ_{org} and ψ_{water} are the association factors of the organic component and water, respectively (ψ =1.9 for methanol and ψ = 2.6 for water) and

MW_{org} and MW_{water} are the molecular weights of the organic component and water, respectively. V_A is the molar volume of the solutes, and these values were obtained from ChemSpider (PPA = 141.006 cm^3, ephedrine = 162.711 cm^3 and methylephedrine = 117.655 cm^3).

Table 2.1 shows the mobile phase compositions employed depending on the pH and column temperature, calculated eluent velocities and diffusion coefficients for the three compounds.

The pressure drop of the column was investigated by subtracting the experimental pressure drop in the connection tubes (ΔP_{ext}) from the total pressure drop from the instrument (ΔP_{tot}) to yield the effective column pressure gradient (ΔP_{col}):

$$\Delta P_{col} = \Delta P_{tot} - \Delta P_{ext}$$

This value was then used in Darcy's law equation to obtain u_0-based column permeability, K_{v0}, calculated from:

$$K_{v0} = \frac{u_0 \eta L}{\Delta P_{col}}$$

where u_0 is the velocity of the unretained component, η is the mobile phase viscosity in cP, ΔP_{col} is the pressure drop across the column in Pa. The system pressure values required for the calculation of ΔP_{col} are determined at each temperature by replacing the column with a zero-volume union. The calculated column permeabilities are 2.41×10^{-15} m^2 for the pH 3 system and 4.08×10^{-15} m^2, 3.88×10^{-15} m^2 and $3.45 \times 10^{-15} m^2$ for the pH 10 systems at 30, 45 and 60 ℃, respectively.

Table 2.1. Physiochemical properties of the employed mobile phase conditions and analytes.

	Volumetric organic factor (%) (η [cP])			**D_{mol} for PPA [10^{-6} cm^2/s]**			**D_{mol} for ephedrine [10^{-6} cm^2/s]**			**D_{mol} for methylephedrine [10^{-6} cm^2/s]**		
	30 °C	45 °C	60 °C	30 °C	45 °C	60 °C	30 °C	45 °C	60 °C	30 °C	45 °C	60 °C
pH 3	0.05 (0.955)	-	-	8.31	-	-	7.32	-	-	8.89	-	-
pH 10	0.2 (1.266)	0.2 (0.931)	0.2 (0.704)	6.34	9.05	12.50	5.81	8.30	11.50	7.07	10.01	14.00

2.4 Results and Discussion

2.4.1 Method development

2.4.1.1 LC Optimisation

Mobile phase pH provides a powerful tool in method development for ionisable compounds, although its use has been largely restricted due to the destructive effects on silica-based packing materials. In addition, the solubility of the analytes in question must also be considered at high pH where they exist in their neutral state. Thus, previous approaches to the analysis of basic analytes have relied upon low pH conditions so as to suppress the ionisation of residual silanol groups or the use of ionic additives and organic modifiers [96, 116, 117]. Unfortunately, such approaches are not without their disadvantages, including reduced retention of hydrophilic bases at low pH and certain mobile phase additives being incompatible with mass spectrometry, such as volatile ion-pairing agents. The chemical stability of new hybrid stationary phase materials, however, facilitates the use of mobile phases of high pH in order to suppress ionisation of basic analytes, reducing ionic interactions with the packing material. The exploitation of pH to render basic compounds in their neutral state results in improved retention and peak shape and, therefore, resolution. These distinct advantages are evaluated here with the separation of the ephedrines which are both hydrophilic (log*P* 0.81-1.74) and basic (pK_a 9.3-9.8).

2.4.2 Effects of organic modifier on apparent pH

The Henderson-Hasselbalch equation describes the derivation of pH as a measure of acidity, using pK_a as the acid dissociation constant:

$$pH = pK_a + log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

where K_a is the acid dissociation constant, pK_a = -log K_a, and [HA] and [A^-] are the molarities of the weak acid and its conjugate base.

The pK_a is a characteristic constant of an analyte. From the above equation it can be seen that by adjusting the mobile phase pH we can vary the relative amounts of neutral and ionic forms of the analyte. If the pH is two units above or below the pK_a of the analyte, more than 99 % of the analyte will either be in its neutral or ionised form, depending on the direction of the pH shift. In the case of a basic compound, as the mobile phase pH is decreased, a larger proportion of the analyte will become ionised, as depicted in the reversed sigmoidal curve in Figure 2.2. Table 2.2 illustrates how mobile phase pH influences the degree of ionisation for the analytes considered in this study. The percentage ionisation may be calculated using the analyte pK_a and specific pH using the following equations:

$$\%\ ionised = \frac{100}{1+10^{(pKa-pH)}} \qquad \text{Acidic}$$

$$\%\ ionised = \frac{100}{1+10^{(pH-pK_a)}} \qquad \text{Basic}$$

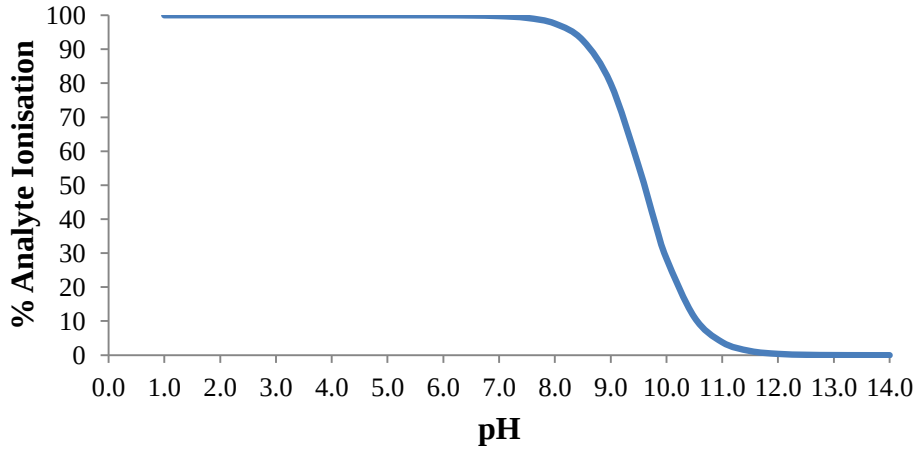


Figure 2.2. Effect of basic analyte ionisation (ephedrine, pK_a = 9.4) as a function of pH.

Table 2.2. Degree of analyte ionisation as a function of pH.

	PPA pK_a 9.4	Cathine pK_a 9.4	Ephedrine pK_a 9.6	Pseudoephedrine pK_a 9.8	Methylephedrine pK_a 9.3
pH	**% ionisation***	**% ionisation***	**% ionisation***	**% ionisation***	**% ionisation***
1.0	100.00	100.00	100.00	100.00	100.00
2.0	100.00	100.00	100.00	100.00	100.00
3.0	100.00	100.00	100.00	100.00	100.00
4.0	100.00	100.00	100.00	100.00	100.00
5.0	100.00	100.00	100.00	100.00	99.99
6.0	99.96	99.96	99.97	99.98	99.95
7.0	99.60	99.60	99.75	99.84	99.50
8.0	96.17	96.17	97.55	98.44	95.23
9.0	71.53	71.53	79.92	86.32	66.61
10.0	20.08	20.08	28.47	38.69	16.63
11.0	2.45	2.45	3.83	5.94	1.96
12.0	0.25	0.25	0.40	0.63	0.20
13.0	0.03	0.03	0.04	0.06	0.02
14.0	0.0	0.0	0.0	0.01	0.00

* % ionisation values calculated from pK_a values using the equation: % ionisation = 100/(1+10^(pH-pKa))

However, an important consideration is the effect of the organic modifier used in RPLC separations on mobile phase pH. It cannot be assumed that the mobile phase pH, and therefore the percentage ionisation, will be the same after the addition of acetonitrile or methanol. The effects of organic content on pH and analyte pK_a have already been investigated and reports demonstrate a negative Δ pH for basic buffers as the methanol content increases [118]. Buffer pH is typically measured in the aqueous phase before the addition of acetonitrile or methanol, using a pH electrode

system calibrated with aqueous buffers. Therefore, although pH measurements of aqueous-organic mixtures will not be accurate, it is useful to measure the apparent pH in order to appreciate the degree of change upon addition of an organic modifier. Figure 2.3 illustrates this apparent change with the addition of methanol or acetonitrile to 10 mM ammonium bicarbonate pH 10, with both solvents causing a decrease in the apparent pH.

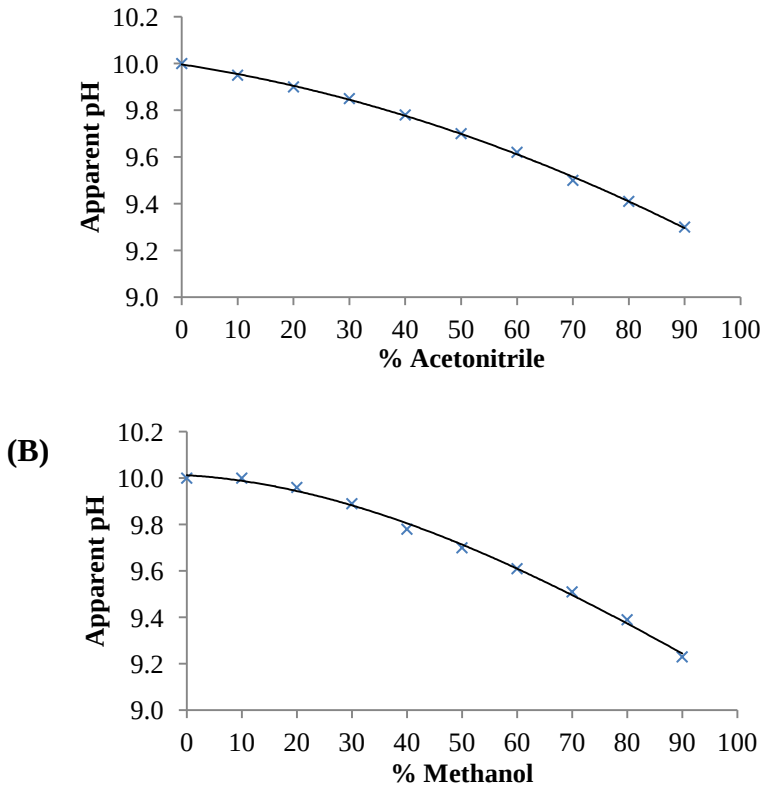


Figure 2.3. Effect of organic modifier using acetonitrile (A) or methanol (B) on the apparent pH of an aqueous 10 mM ammonium bicarbonate buffer solution adjusted to pH 10.

2.4.2.1 Retention

Initial method development investigates the effects of low, mid and high pH mobile phases on peak shape, retention, and hence separation of the ephedrines (PPA, cathine, ephedrine, pseudoephedrine and methylephedrine) in order to determine the

optimum conditions under which to separate the diastereoisomers. Isocratic elution at 0.4 mL/min was performed with varying percentages of organic modifier, investigating the use of acetonitrile and methanol with an aqueous mobile phase buffered to the extremes of pH used, i.e. pH 3 and pH 10. The results are detailed in Figure 2.4 and show the effect of organic modifier on the retention of PPA, ephedrine and methylephedrine under low and high pH conditions.

It is interesting to note that, as the organic content reaches 70-80 %, retention begins to increase again. This phenomenon contradicts traditional RPLC theory, where an increase in organic content dictates a decrease in retention. The differences seen here can be explained by the fact that the phase is subject to operation in a hydrophilic interaction liquid chromatography (HILIC)-type mode. This HILIC behaviour has been reported elsewhere, where Tannak *et al.* showed the HILIC-like properties of cyano, butyl and phenyl HPLC phases for the separation of some basic analytes, denoted by an increase in retention of polar basic analytes as a function of increased organic modifier [119]. This effect is more pronounced at pH 3 than at pH 10, which can be attributed to the ionisation of basic analytes and silanol groups in the stationary phase at low pH whereas, at pH 10, the analytes are largely in their neutral form. When both are ionised, strong ionic interactions are possible and formation of the water layer thought to be responsible for partitioning in HILIC is maximised. In this instance, at pH 10 the basic analytes are largely unionised and are therefore less able to take part in ionic interactions. The effect is more pronounced with acetonitrile as the organic modifier as opposed to methanol, as acetonitrile is a weaker solvent in HILIC mode. Under basic conditions at 90 % acetonitrile, a change in the order of elution starts to reflect the elution order seen in HILIC, with methylephedrine

exhibiting weaker retention than the more hydrophilic analyte, ephedrine (Figure 2.4).

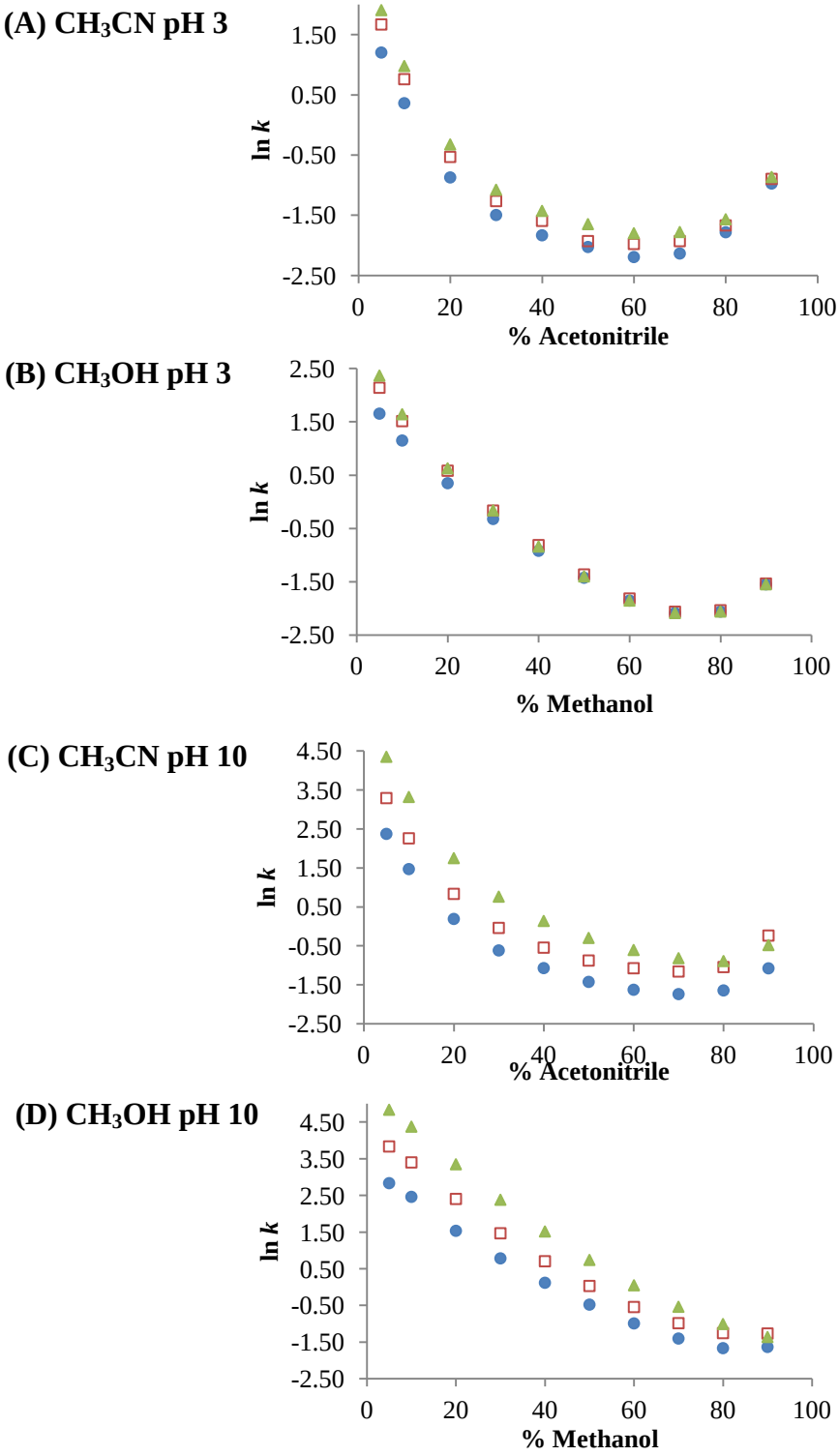


Figure 2.4. Relationship between retention and amount of organic modifier.
Eluent conditions were acidic (pH 3) with acetonitrile (A) and methanol (B) and alkaline (pH 10) with acetonitrile (C) and methanol (D) for PPA (●), ephedrine (□), and methylephedrine (▲), performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

As seen in Figure 2.4, the retention characteristics of three analytes, PPA, ephedrine and methylephedrine, demonstrate poor resolution under low pH conditions compared with complete separation at pH 10. In addition, better resolution was achieved with a higher amount of the weaker organic modifier, methanol, compared to acetonitrile; this solvent system was therefore chosen for future investigations.

2.4.2.2 Effect of pH on retention of basic analytes

Subsequent experiments were performed using methanol as the organic modifier at a ratio of 20:80 v/v CH_3OH: aqueous buffer, using either ammonium formate (10 mM, pH 3, 4, 5), ammonium acetate (10 mM, pH 6, 7) or ammonium bicarbonate (10 mM, pH 8, 9, 10). Although these analytes are not fully unionised until pH 11.8 (2 pH untis above the pK_a value), the highest pH evaluated was pH 10 since dissolution of the stationary phase and analyte solubility were a concern. Figure 2.5 illustrates the relationship between pH and analyte retention for PPA, ephedrine and methylephedrine. Between pH 3 and 7, retention of the analytes is low, resulting in co-elution. As the mobile phase pH is increased to pH 8, we begin to see an increase in analyte retention as the degree of analyte ionisation starts to decrease. Suppression of the ionisation of these hydrophilic bases at high pH enhances retention on the reversed-phase column by facilitating hydrophobic interactions between the analyte and C_{18} ligands of the stationary phase. As pH is increased above the pK_a values of the analytes, a significant increase in retention is exhibited allowing good separation of the analytes. The resulting suppression in analyte ionisation is sufficient to enhance retention to permit good separation, despite the partial degree of ionisation still apparent at these pH values.

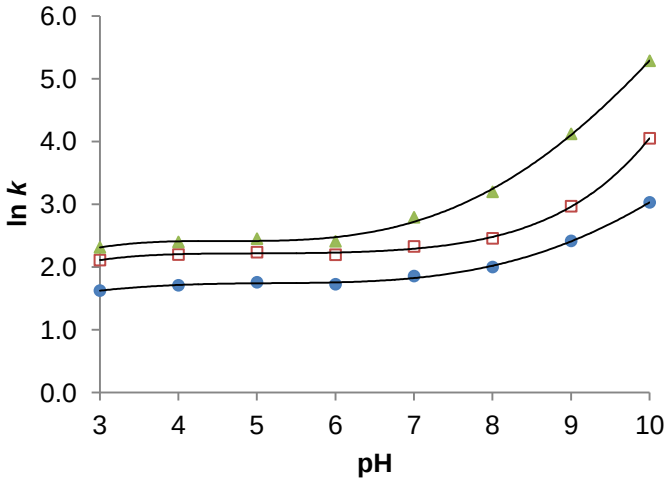


Figure 2.5. Relationship between pH and analyte retention (ln *k*).
Phenylpropanolamine (●) ephedrine (□), and methylephedrine (▲) with aqueous buffer:CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

2.4.2.3 Effect of mobile phase pH on selectivity

Using mass spectrometry to track the peaks, a mix of the five ephedrines was injected under the extreme pH conditions of pH 3 and pH 10 to demonstrate the impact of pH on selectivity. The chromatograms in Figure 2.6 demonstrate the poor retention and co-elution under acidic conditions compared with the substantial increase in retention and resolution between each of the analytes, including the diastereoisomeric pairs, at pH 10. However, peak fronting is noted for PPA and cathine under high pH conditions despite using an injection solvent matched to the starting mobile phase conditions. This phenomenon is possibly related to the solubility of these analytes at high pH.

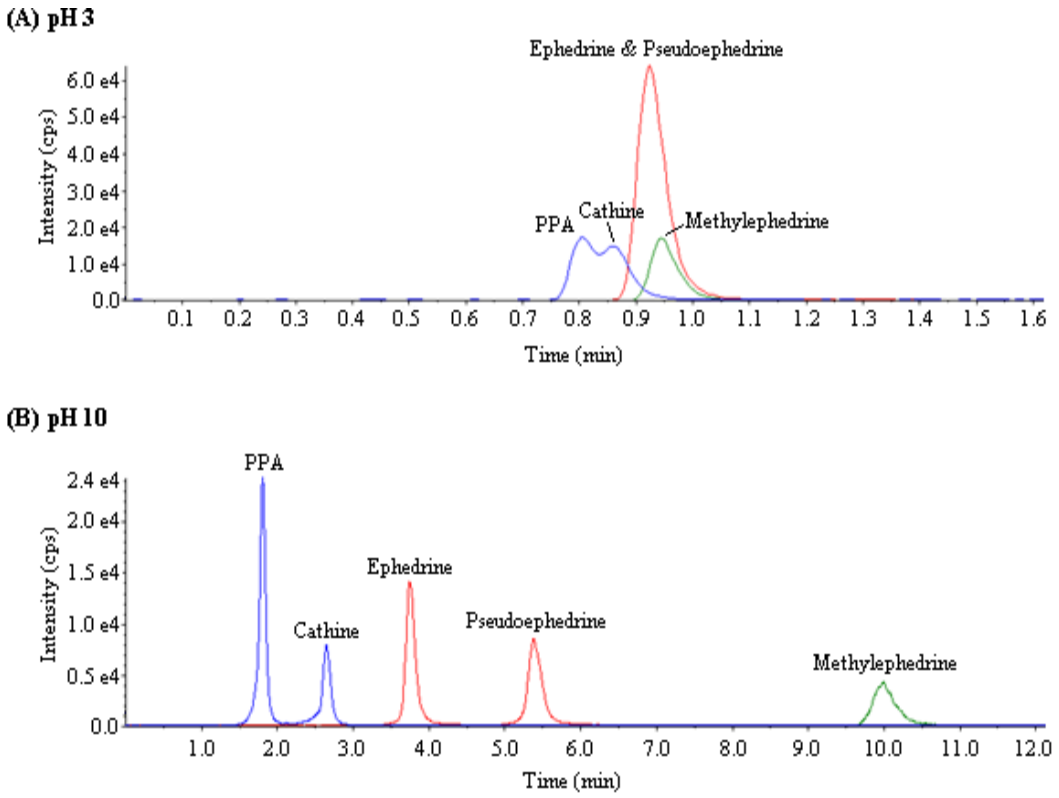


Figure 2.6. Effect of mobile phase pH on selectivity.
Isocratic chromatography was performed with 10 mM ammonium formate pH 3 (A) and 10 mM ammonium bicarbonate pH 10 (B) and CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

2.4.2.4 Peak shape

Peak tailing is also greatly improved at pH 10 compared to pH 3 or 7. With 0.1 µg of material loaded onto the column, isocratic elution of pseudoephedrine at pH 3, 7 and 10 generated peaks with asymmetry factors of 3.95, 4.75 and 1.96, respectively, calculated at 10 % peak height. Peak tailing was most severe at pH 7, where both the residual silanol groups of the stationary phase and basic analytes are fully ionised and thus ionic interactions between the two are greatest. At pH 3, peak tailing was only slightly reduced, since the pH is not low enough to eliminate silanol activity. The benefit of increasing the pH appears at pH 10. Although pH 10 is not sufficient to completely eliminate interactions with the sorbent surface, it has been clearly demonstrated to dramatically reduce them, with evident benefits for chromatography.

2.4.2.5 Loading

Overloading of ionised bases on silica-based phases occurs readily at low pH, a phenomenon that has been widely discussed in literature. The exploitation of high pH mobile phases has previously been reported to enhance the sample loading capacity of basic analytes [104-107]. Here, the loading capacity at low, mid and high pH is evaluated for pseudoephedrine, with the overlaid chromatograms in Figure 2.7 illustrating the beneficial effects of increasing mobile phase pH.

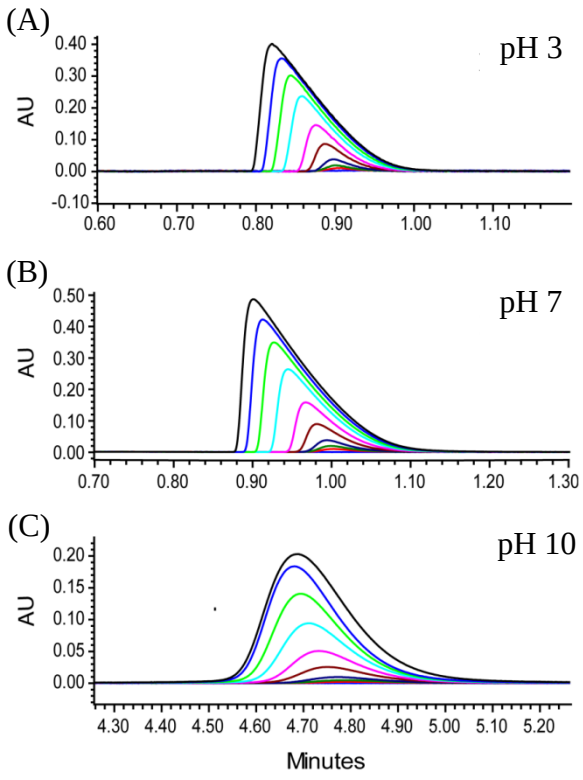


Figure 2.7. Effect pH on chromatography with of increasing analyte concentration.
Overlaid chromatograms of 5-500 ng pseudoephedrine using mobile phases at the following pH values: pH 3 (A); pH 7 (B); pH 10 (C) with aqueous buffer: CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

Increasing the sample mass from 5-500 ng demonstrates rapid deterioration of peak shape at pH 3 and 7, whereas at high pH a greater sample mass is tolerated before sample overloading becomes apparent (Figure 2.8). Reduced protonation, even if not complete, through the use of high pH has previously been documented to improve sample loading capacity, for which several explanations have been proposed. Given the reduced silanol activity of new generation phases, it is likely that reduced analyte ionisation suppresses mutual repulsion of protonated species held on the surface of the stationary phase. Alternatively, charged species may simply be unable to fully penetrate the stationary phase, therefore by reducing analyte protonation the capacity for interaction with the hydrophobic ligands is increased.

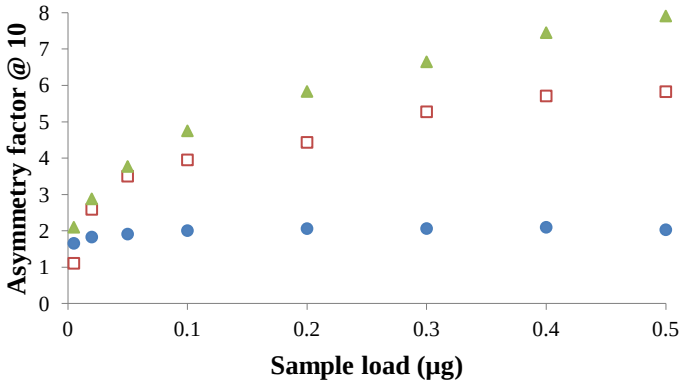


Figure 2.8. Effect of sample mass on peak asymmetry (A_s at 10 % peak height). Values obtained at pH 3 (□), pH 7 (▲) and pH 10 (●) with aqueous buffer:CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

The ability to tolerate larger sample loading capacities is also reflected in the retention times, which are prone to decreasing as sample mass is increased in an overload situation as the apex of the peak shifts to the left. This is depicted in Figure 2.9, where the retention factor shift at pH 10 is small as the amount of sample increases, whereas at pH 3 and 7 the slope of the retention plot shows a decrease in retention with increased sample mass.

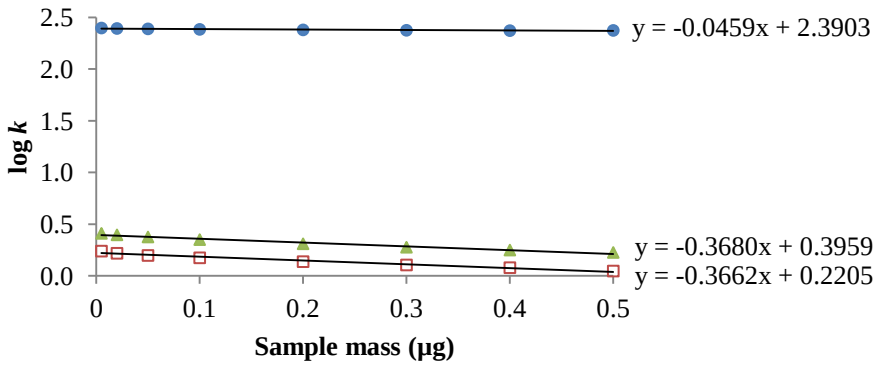


Figure 2.9. Effect of sample mass on retention factor (log *k*). Values obtained at pH 3 (□), pH 7 (▲) and pH 10 (●) with aqueous buffer:CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

Figure 2.10 portrays the relationship between column performance and increased sample mass under the three pH conditions, showing the ability to maintain high efficiency separations as sample mass is increased at pH 10, whereas at pH 3 and 7 the column efficiency rapidly deteriorates. The additional benefit of operating at high pH with these basic compounds is the ability to simultaneously analyse compounds present at varying degrees of concentration; for instance being able to detect a low level analyte together with an analyte present at a much greater concentration without any problems with overloading.

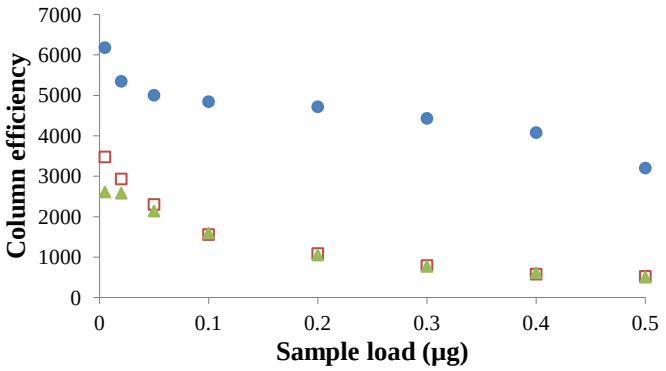


Figure 2.10. Effect of sample load on column efficiency N.
Conditions were pH 3 (□), pH 7 (▲) and pH 10 (●) with aqueous buffer: CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

2.4.3 Kinetic evaluation under acidic and basic conditions

2.4.3.1 Van Deemter

Peak efficiency is typically compromised for basic analytes which exhibit secondary ionic interactions causing peak distortion. Here, the same stationary phase support is used to investigate the influence of pH on column performance through analysing basic analytes in their neutral and protonated states. Van Deemter curves were constructed for PPA, ephedrine and methylephedrine in acidic (pH 3) and basic

(pH 10) eluent conditions. Duplicate injections were averaged at each linear velocity, performed in the isocratic mode. The organic content of the mobile phase was adjusted in an attempt to maintain constant retention factors between the two sets of conditions.

The actual data, represented by markers, and the best fit curves to the van Deemter equation, are displayed for PPA, ephedrine and methylephedrine at both pH 3 and pH 10 (Figure 2.11). Curve fitting was performed using SigmaPlot 12 on the *H* versus u_0 data in order to determine the best *A*, *B* and *C* values of the van Deemter equation. Figure 2.11 shows the difference in performance between pH 3 and pH 10, with pH 10 indicating significantly better efficiency for ephedrine and methylephedrine. These coefficients, together with u_{opt} and H_{min} values for each analyte under pH 3 and pH 10 conditions, are given in Table 2.3. At pH 3, the *A*, *B* and *C* coefficients are all similar for each analyte. At pH 10, significantly lower H_{min} values are gained for ephedrine and pseudoephedrine, while PPA does not seem to follow this trend. It is proposed that the poor efficiency and poor fit to the curve for PPA is due to the fronting peak shape exhibited by PPA at high pH (outliers in Figure 2.11 (A) circled). This could be explained by poor solubility of this compound under pH 10 conditions at 30 °C, and it is seen to improve with increased temperature, indicating that solubility increases with temperature. The improvement in chromatographic performance for ephedrine and methylephedrine at high pH is a consequence of the suppression of secondary interactions with the stationary phase.

Table 2.3. Values for van Deemter coefficients.

Analyte	*k*	*A*	*B*	*C*	u_{opt} (mm/s)	H_{min} (µm)
pH 3 30 °C						
PPA	5.07	8.23	1.00	0.61	1.29	9.78
Ephedrine	8.23	7.34	1.36	0.69	1.41	9.29
Methylephedrine	10.1	7.23	1.61	0.63	1.58	9.25
pH 10 30 °C						
PPA	4.35	7.09	11.5	0.48	4.87	11.8
Ephedrine	9.49	2.83	3.06	0.80	1.95	5.97
Methylephedrine	26.6	1.43	4.77	0.76	2.51	5.25

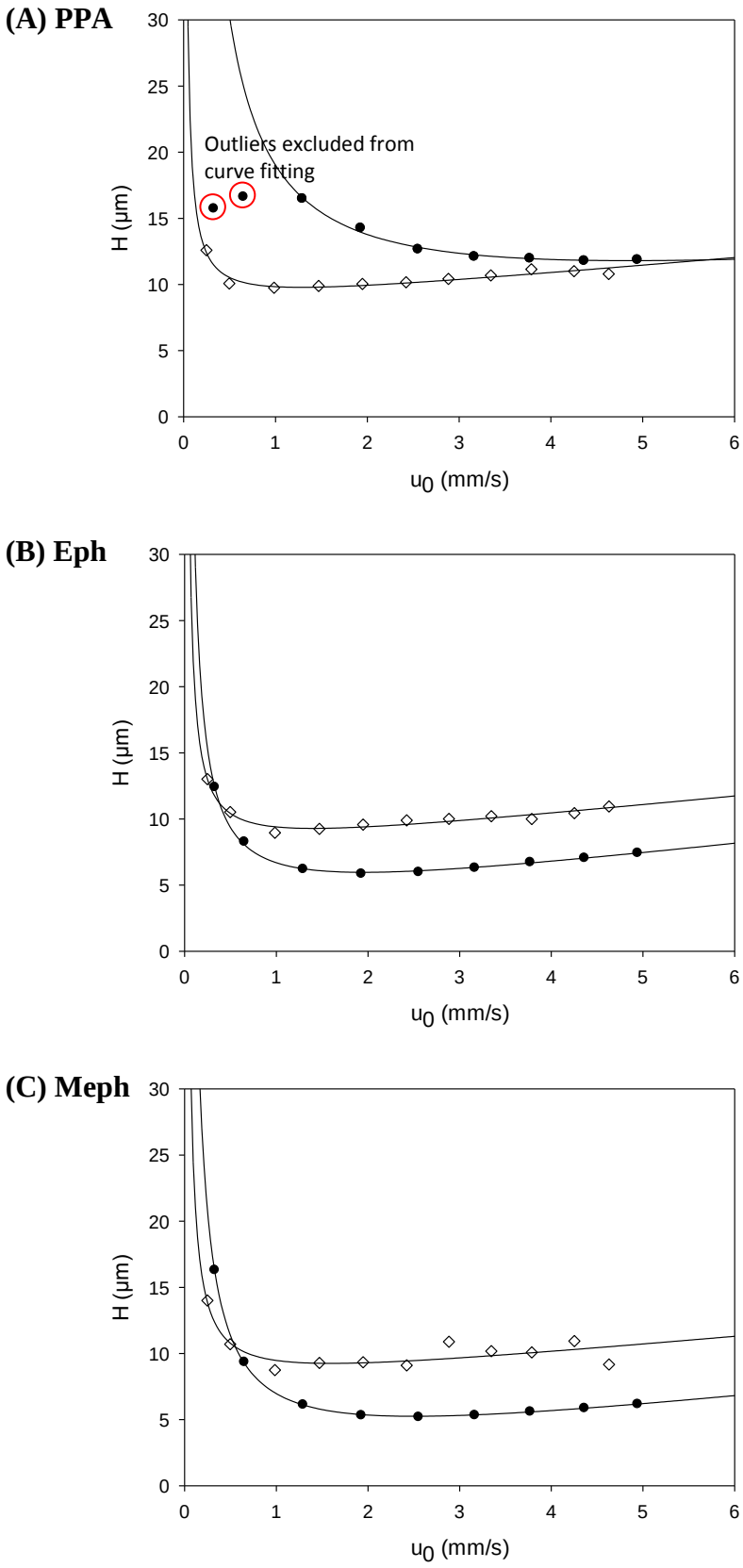


Figure 2.11. van Deemter plots for PPA (A), ephedrine (B) and methylephedrine (C) under pH 3 95:5 v/v aqueous buffer: CH_3OH (◊) and pH 10 80:20 v/v aqueous buffer: CH_3OH conditions (●) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column, 30 °C.

2.4.3.2 Kinetic plots

The data generated from the previous van Deemter experiments was further transformed to obtain kinetic plots. Kinetic plots are a useful tool for comparing the performance of different chromatographic systems, in this case the effect of pH on the analysis of basic analytes, exemplified by PPA, ephedrine and methylephedrine. The kinetic plot method can also be used to illustrate the shortest analysis time possible to achieve a certain number of theoretical plates. The method uses the two equations below to translate the (H, u_0) van Deemter data into (N, t_0), calculated at the maximum obtainable column pressure, ΔP_{max}:

$$N = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{v0}}{u_0 H}\right]_{exp}$$

and

$$t_0 = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{v0}}{u_0^2}\right]_{exp}$$

where the viscosity (η) and column permeability (K_{v0}) are calculated and the plate height (H) and linear velocity (u_0) are experimentally obtained values, generated with one 5 cm column at each mobile phase at pH 3 and 10. Figure 2.12 shows a plot of the total measured pressure drop as a function of the flow rate, illustrating the higher back-pressure generated by the basic eluent due to its higher viscosity.

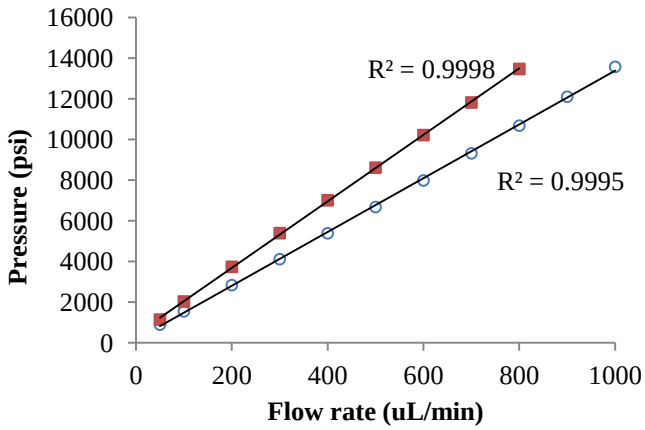


Figure 2.12. Experimental pressure drops at different flow rates.
Measurements taken under the acidic (○ pH 3 95:5 v/v aqueous buffer: CH_3OH) and basic (■ pH 10 80:20 v/v aqueous buffer: CH_3OH) conditions used in this study performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column, 30 °C.

There are several members of the kinetic plot family used to represent and compare performance limits of different chromatographic systems. The plot of t_0 versus N is the simplest tool for comparing column performance under different chromatographic conditions, in this case the evaluation of different pH eluents (Figure 2.13). This plot directly shows the range of plate numbers where the high pH system can yield faster separation. For fast separations, the high pH system produces a greater number of plates compared with the low pH system for a given dead time. Figure 2.13 indicates that it would take approximately three times longer to achieve a required plate number with the acidic eluent compared with the basic eluent. As an example, to generate 30,000 plates under acidic conditions an analysis time of 7.5 min would be required, compared with 2.5 min under basic conditions. However, as the analysis time increases, the performance of the columns becomes similar.

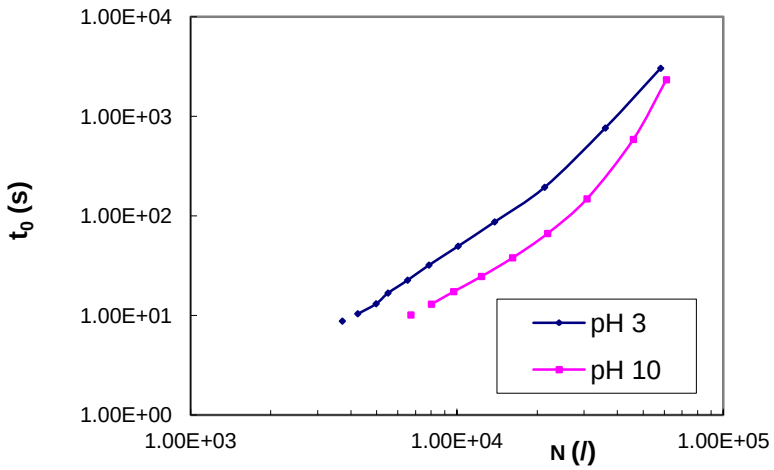


Figure 2.13. Kinetic plots of analysis time (t_0) *versus* efficiency (N).
Generated from the van Deemter data of the separation of ephedrine under acidic (pH 3 95:5 v/v aqueous buffer: CH_3OH) and basic (pH 10 80:20 v/v aqueous buffer: CH_3OH) conditions performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column, 30 °C.

The following impedance time plot (Figure 2.14) has several advantages over the t_0 versus N plot, since it yields the most expanded y-scale and immediately shows which plate number (referred to as N_{opt}) a given system achieves its most advantageous kinetic performance over pressure cost ratio. The axis of the N-axis is reversed to resemble the familiar van Deemter-curve (B-region to the left and C-region to the right). Although the number of achievable theoretical plates remains the same under acidic and basic conditions, the curve obtained at pH 10 is much lower than that obtained at pH 3. The number of theoretical plates generated when operated at their optimum conditions (N_{opt}) is the same, but the analysis time and pressure required to achieve N_{opt} is greater under acidic conditions. Basically, higher efficiencies (N) can be generated with a shorter retention time and lower pressure drop at pH 10.

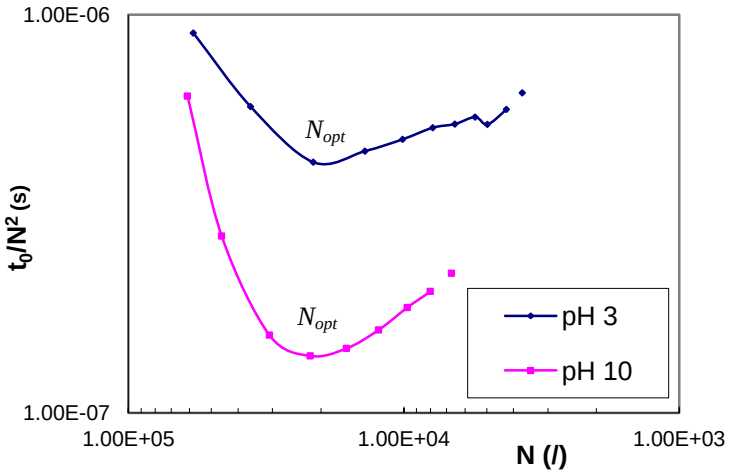


Figure 2.14. Kinetic plots of impedance (t_0/N^2) *versus* efficiency (N). Generated from the van Deemter data of the separation of ephedrine under acidic (pH 3 95:5 v/v aqueous buffer: CH_3OH) and basic (pH 10 80:20 v/v aqueous buffer: CH_3OH) conditions performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column, 30 °C.

The (u_0, H) plots can also be readily transformed into plots of analysis time *versus* resolution to evaluate differences in selectivity that exist among systems with differing stationary or mobile phase properties. Resolution plots were constructed for both eluents by calculating the resolution of ephedrine and methylephedrine at every flow rate. From Figure 2.15, it is clearly evident that greater resolution of the critical pair can be achieved at pH 10 compared with pH 3, despite a shorter analysis time. For the same analysis time, resolution of the critical pair will always be greater at pH 10 compared with pH 3, for example for an analysis time of 100 s, the resolution between the two analytes is 2.8 at pH 3 and 12 at pH 10. Sample chromatograms obtained under the two sets of conditions at the same flow rate (0.4 mL/min) are presented in Figure 2.16.

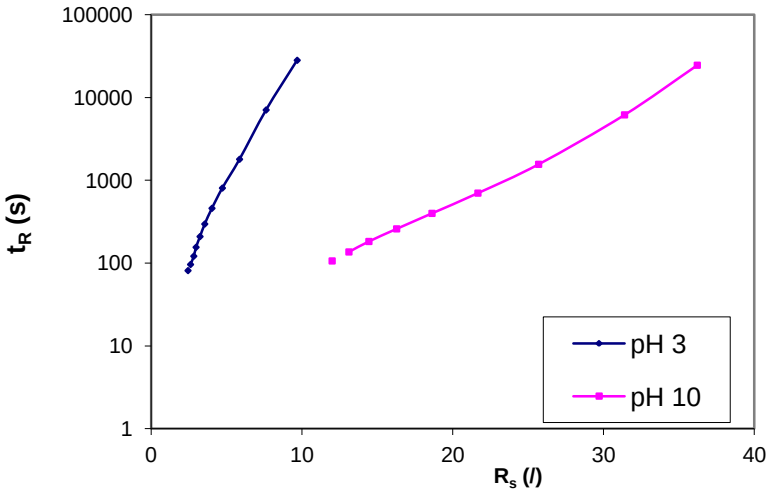


Figure 2.15. Plots of analysis time *versus* resolution (*Rs*).
Generated for ephedrine and methylephedrine under acidic (pH 3 95:5 v/v aqueous buffer: CH_3OH) and basic (pH 10 80:20 v/v aqueous buffer: CH_3OH) conditions performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column, 30 °C.

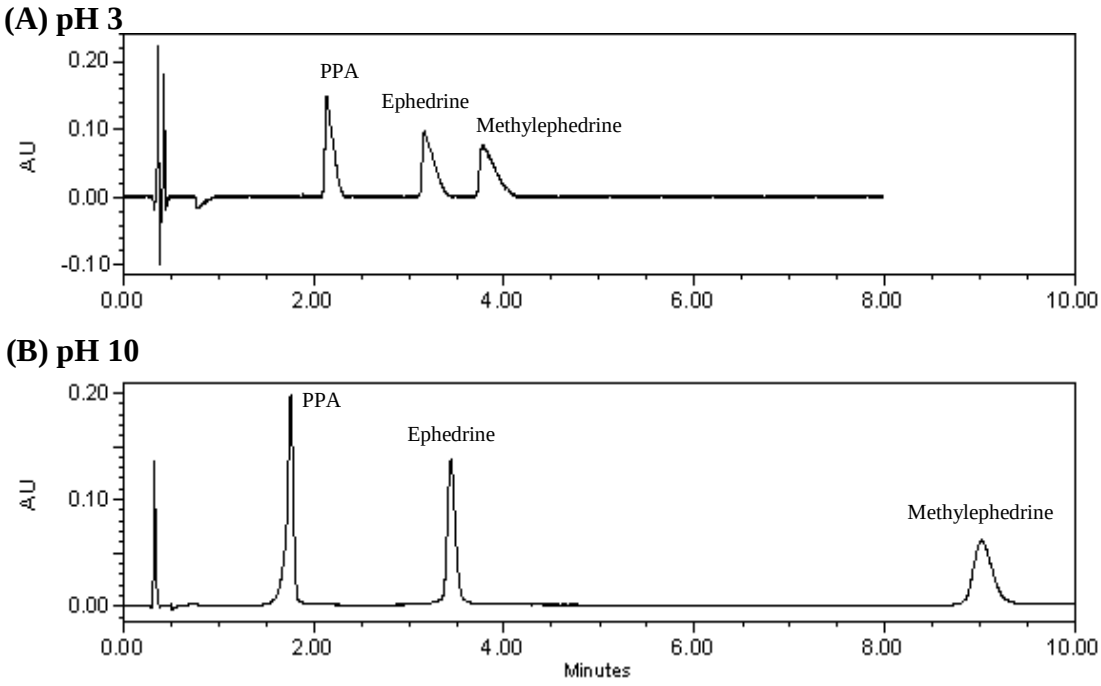


Figure 2.16. Chromatograms of PPA, ephedrine and methylephedrine acidic (pH 3 95:5 v/v aqueous buffer: CH_3OH) and basic (pH 10 80:20 v/v aqueous buffer: CH_3OH) conditions performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min, 30 °C.

2.4.4 Increasing temperature with high pH mobile phase

2.4.4.1 Effect of increasing temperature on retention

Temperature is another key variable in LC, with the potential to influence retention, selectivity, peak shape and efficiency [7, 66, 112-114, 120, 121]. Where high-throughput, high productivity and high resolution are of great interest in pharmaceutical analysis, temperature has been shown to be a powerful tool in fulfilling these requirements on conventional instrumentation. The following data details the benefits of increasing column temperature with a basic chromatographic eluent, impacting on retention, analysis time, peak shape and column efficiency (Figure 2.17). Initial method development was performed at a column temperature of 30 °C. The effects of temperature were then investigated while maintaining the same chromatographic conditions and making increments of 5 °C in the column temperature. All separations were performed in the isocratic mode using a mobile phase composition of 80:20 v/v ammonium bicarbonate: CH_3OH.

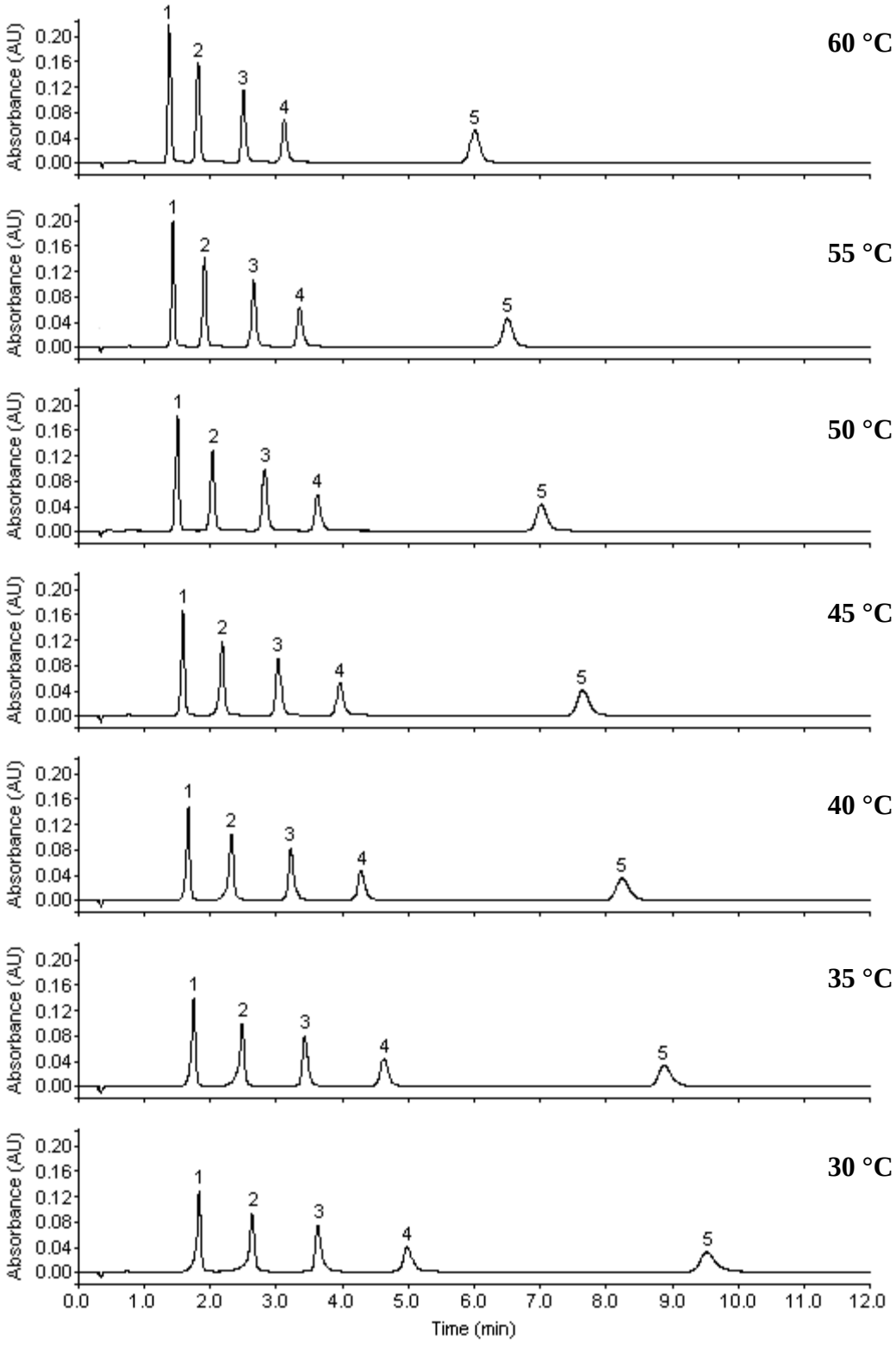


Figure 2.17. Effect of column temperature on chromatography.
PPA (1), cathine (2), ephedrine (3), pseudoephedrine (4) and methylephedrine (5) with 10 mM ammonium bicarbonate pH 10: CH_3OH (80:20 v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min.

The Wilke-Chang equation describes the relationship between the diffusion coefficient, solvent viscosity and absolute temperature [115]. An increase in column temperature elicits an increase in the diffusion of analytes in the mobile and stationary phase, and reduced mobile phase viscosity which also enhances analyte diffusivity. Significant reductions in back-pressure are seen with increased temperature through reduced viscosity. This feature can be exploited to obtain faster analyses by permitting increased flow rates or to generate higher plate numbers by using longer or coupled columns [7, 114]. With respect to the van Deemter equation, the *B*- and *C*-terms are temperature dependent; the *B*-term being directly proportional and the *C*-term inversely proportional to the diffusion coefficient. It has been shown that elevated temperature has a significant effect on the shape of van Deemter curves, with a shift in u_{opt} and a flatter curve seen at higher velocities. Higher flow rates can therefore be employed at elevated temperatures to gain faster analyses without sacrificing efficiency. Whether the use of temperature can be employed to produce a gain in efficiency has been debated and no absolute increase in efficiency can be attributed to an increase in temperature alone. An improvement in efficiency will only be achieved if separations are performed under sub-optimal conditions, since the drawbacks of operating a system above its optimal linear velocity is offset at elevated temperature.

The van't Hoff equation can be used to describe the relationship between temperature and solute retention factor:

$$Log\ (k) = -\frac{\Delta H_0}{2.3RT} - \frac{\Delta S_0}{2.3R} - \log ø$$

where ΔH_0 and ΔS_0 are the enthalpy and entropy of solute transfer, respectively, from the mobile phase to the stationary phase, T is the absolute temperature, R is the universal gas constant and $ø$ is the phase ratio of the column. Here, the ephedrines all show linear van't Hoff behaviour, displaying a decrease in retention with increased column temperature (Figure 2.18), indicating no change in selectivity for these analytes.

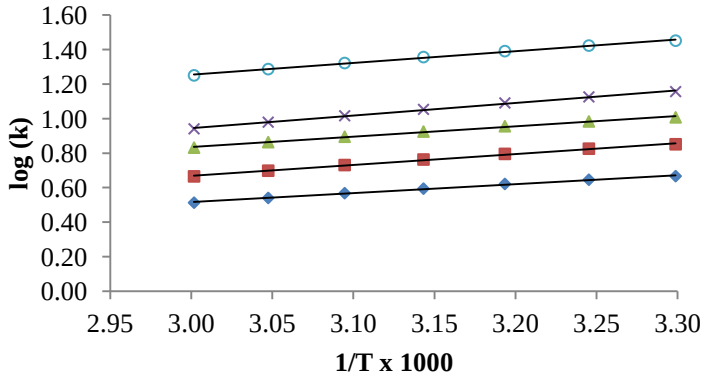


Figure 2.18. Variation of log (*k*) versus 1/*T*.
PPA (♦), cathine (■), ephedrine (▲), pseudoephedrine (x) and methylephedrine (○) at 50 µg/mL using 10 mM ammonium bicarbonate pH 10 and methanol (80:20, v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min.

Resolution is often compromised in favour of speed where high-throughput analyses are required. Here, resolution between the two pairs of diastereoisomers, PPA-cathine and ephedrine-pseudoephedrine, is affected by the increase in temperature with a slight reduction in *Rs* values from 7.0 to 5.3 and 7.0 to 5.1, respectively, at

30 °C and 60 °C (Figure 2.17). A change in temperature does not, however, influence resolution between cathine and ephedrine or pseudoephedrine and methylephedrine.

Improvements in peak shape are also brought about by increasing temperature. This is clearly seen in Figure 2.17 and Figure 2.19, with a particularly dramatic improvement seen for the primary amines, PPA and cathine, which exhibit significant peak fronting at lower temperatures. For example, $As_{10\%}$ for PPA is 0.52 at 30 °C compared with 1.04 at 60 °C. This aforementioned peak fronting can be explained by poor solubility of these analytes in high pH conditions, where they exist in their largely neutral forms, which is improved at higher temperatures. The slight tailing which is still apparent for the secondary (ephedrine and pseudoephedrine) and tertiary (methylephedrine) amines at pH 10 is also reduced as temperature is increased, with $As_{10\%}$ values all close to 1.0 at 60 °C. In addition to the more Gaussian peak shape obtained, peak widths are also affected by increased temperature. Due to faster kinetics, peak width decreases as temperature is increased, while peak area remains constant, thereby peaks inherently become sharper to give greater sensitivity.

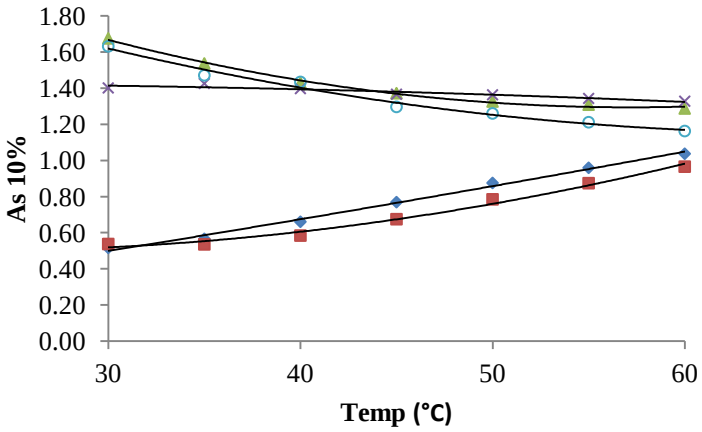


Figure 2.19. Effect of temperature on peak asymmetry (As 10 % peak height). PPA (♦), cathine (■), ephedrine (▲), pseudoephedrine (x) and methylephedrine (○) at 50 µg/mL in 10 mM ammonium bicarbonate pH 10 and methanol (80:20, v/v) performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column at 0.4 mL/min.

2.4.4.2 Effect of varying temperature on kinetic performance

Van Deemter plots were constructed from experimentally obtained H and u_0 values for PPA, ephedrine and methylephedrine under pH 10 conditions at 30, 45 and 60 °C (Figure 2.20). These plots illustrate the influence of temperature on column efficiency, displaying flatter van Deemter curves when the temperature was increased. The Chen-Horvath equation explains the relationship between temperature and mobile phase viscosity and this can be visualised in Figure 2.21 with a reduction in back-pressure as temperature increases, allowing faster flow rates to be realised. Optimal linear velocity (u_0) is therefore shifted to higher values, although the optimal plate height remains virtually unaffected. This phenomenon has been extensively investigated, with Horvath reporting that higher temperatures generate higher optimal linear velocities and flatter curves, but that minimum plate height is not affected for small molecules with fast sorption kinetics [122]. The poor curve fitting noted for PPA (Figure 2.11 (A)) at 30 and 45 °C is a result of peak fronting, thought to be due to poor analyte solubility at pH 10. As previously discussed, the

extent of this peak fronting diminishes with an increase in temperature, which explains the more typical curve generated for PPA at 60 °C. As detailed in Table 2.4, with the exception of PPA because of the poor curve fitting, u_{opt} increases with an increase in temperature. For example, for ephedrine u_{opt} at 30 °C is 1.95 mm/s compared with 3.66 mm/s at 60 °C. H_{min}, on the other hand, are not subject to any significant change as temperature is increased (Table 2.4).

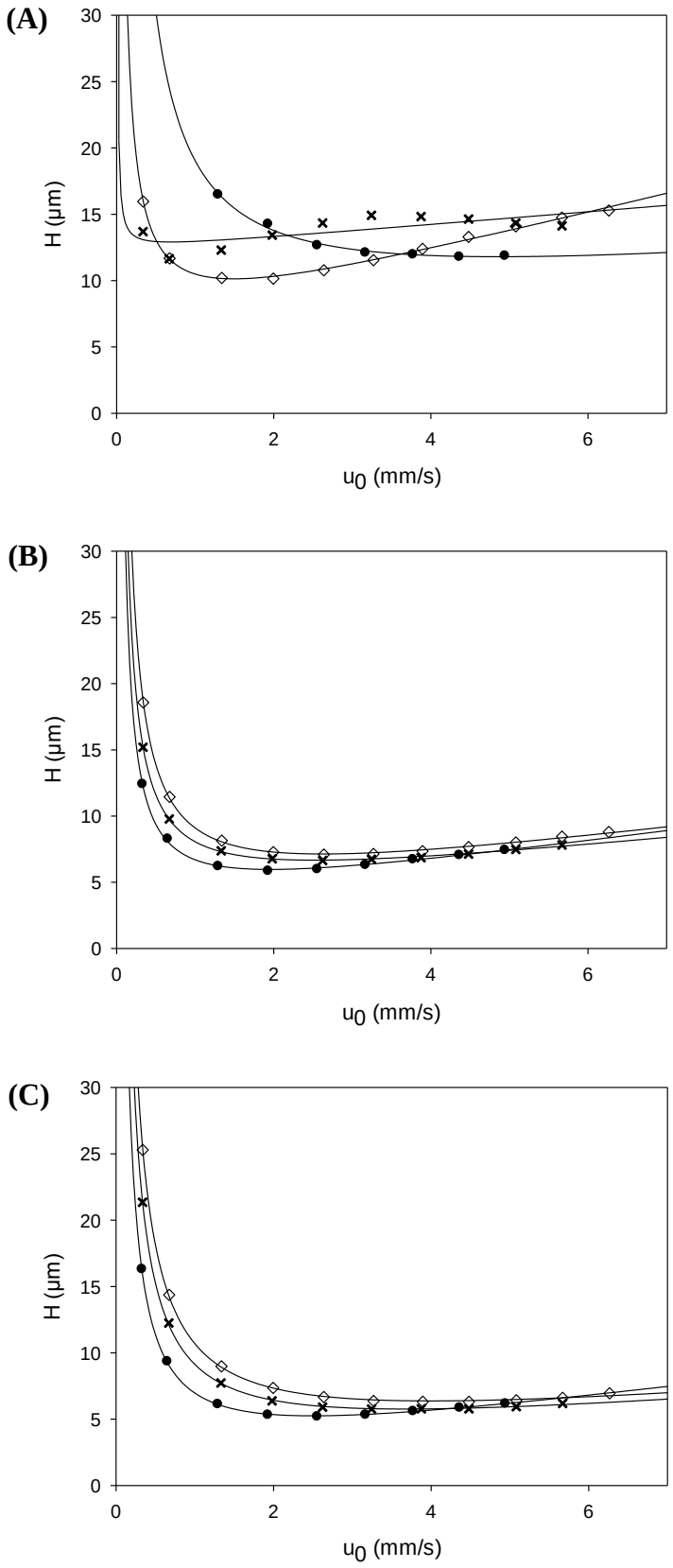


Figure 2.20. van Deemter curve for PPA (A), ephedrine (B) and methylephedrine (C) under pH 10 conditions (80:20, v/v aqueous buffer:CH_3OH) at 30 (•), 45 (×) and 60°C (◊), performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column.

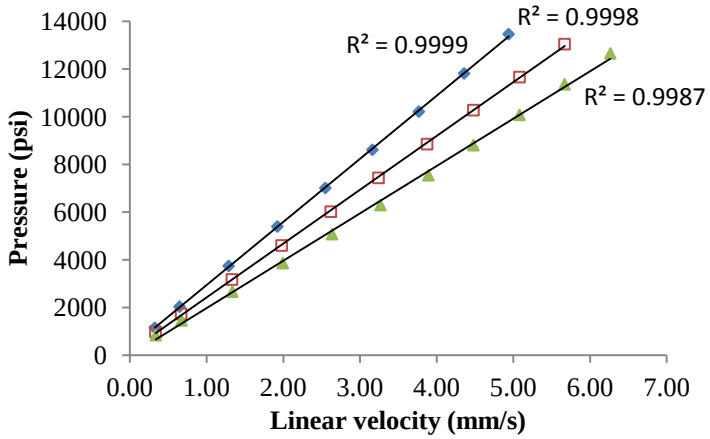


Figure 2.21. Relationship between temperature and back-pressure at 30 °C(♦), 45 °C (□) and 60 °C (▲).

Table 2.4. Values for van Deemter coefficients determined under pH 10 conditions at 30, 45 and 60 °C.

Analyte	*k*	*A*	*B*	*C*	u_{opt} (mm/s)	H_{min} (µm)
PPA						
30 °C	4.35	7.09	11.5	0.48	4.87	11.8
45 °C	3.95	12.2	0.23	0.49	0.68	12.9
60 °C	3.26	5.69	3.31	1.49	1.50	10.1
Ephedrine						
30 °C	9.49	2.83	3.06	0.80	1.95	5.97
45 °C	8.48	3.64	3.82	0.60	2.52	6.67
60 °C	6.79	3.22	5.13	0.75	3.66	5.76
Methylephedrine						
30 °C	26.6	1.43	4.77	0.76	2.51	5.25
45 °C	23.0	2.30	6.35	0.47	2.63	7.13
60 °C	17.8	2.55	7.66	0.48	3.99	6.38

2.4.4.3 Comparison of HPLC and UHPLC

The trend in decreasing particle size affords several important advantages in separation science by maximising column efficiency. The chromatogram in Figure 2.22 illustrates the gain in efficiency by reducing the particle size of the same column chemistry while keeping all other parameters constant. Under the conditions

tested, for the most retained analyte, methylephedrine, 5418 plates on-column are generated on the 2.5 µm material, while the 1.7 µm variant generates 9141 plates on column. It should be noted, however, that the peak shape between the two column formats is not the same, with the fronting PPA and cathine peaks not as pronounced with the 2.7 µm material, while a greater degree of tailing is exhibited for ephedrine and pseudoephedrine. Although both columns were tested with a neutral test mix prior to this study to confirm good chromatographic performance, the 2.7 µm phase appears to have more exposed silanols on the surface, giving a greater degree of secondary interactions. However, such benefits in chromatographic performance realised with UHPLC technologies allow chromatographers to further improve separations and speeds of analysis with inherent enhanced resolution and gains in sensitivity with sharper peaks.

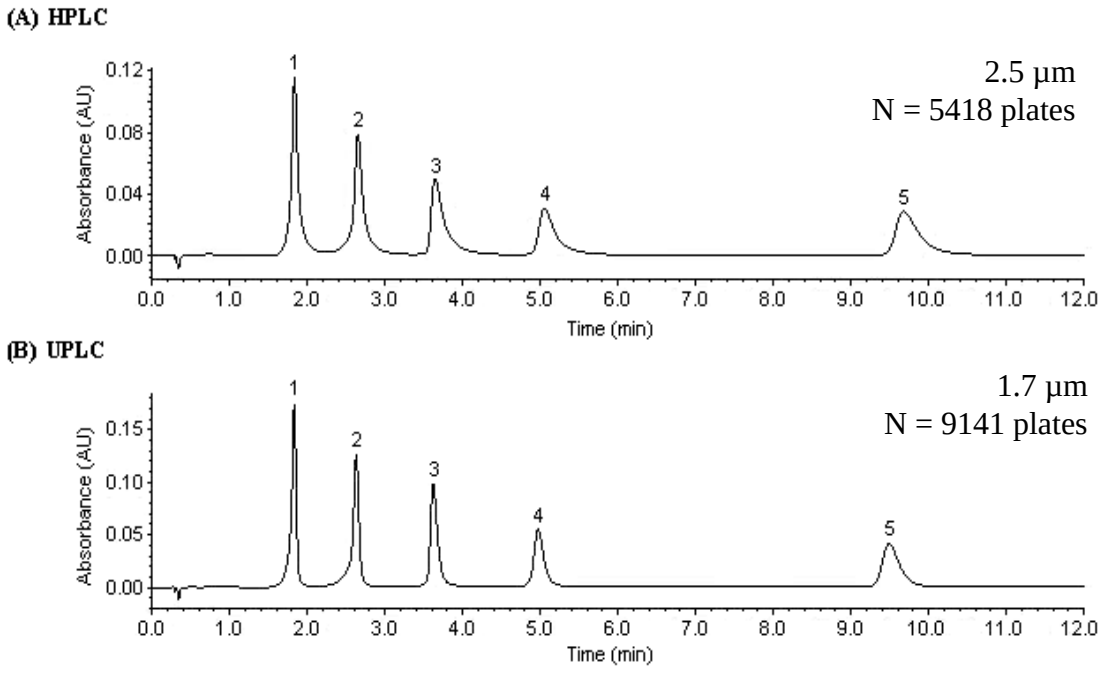


Figure 2.22. Comparison of HPLC (A) and UPLC® (B).
Separation of PPA (1), cathine (2), ephedrine (3), pseudoephedrine (4) and methylephedrine (5) on an XBridge 2.7 µm, 50mm x 2.1 mm i.d. (A) compared with an Acquity BEH 1.7 µm, 50 mm x 2.1 mm (B) columns under isocratic elution with 80:20 v/v pH 10 ammonium bicarbonate:CH_3OH at 0.4 mL/min.

2.4.4.4 Final conditions

Although the benefits of using an elevated mobile phase pH and column temperature have been clearly illustrated, these parameters are limited by the chemical stability of stationary phase materials. During this method development, several columns started to exhibit poor efficiencies, severely tailing peaks and split peaks shortly after use with high pH eluents. Therefore, in order to preserve column lifetime, the final mobile phase conditions comprised ammonium bicarbonate buffered to pH 9.8 which, although a proportion of the analyte will remain ionised, was found to be sufficient for good resolution and peak shape. Similarly, elevated temperature accelerates dissolution of the stationary phase, despite the enhanced stability of the hybrid material. Therefore, a temperature of 45 °C was chosen as a compromise between the benefits of increased temperature and preserving column lifetime. Despite the knowledge that smaller particles generate higher column efficiencies and provide faster analysis without sacrificing the quality of a separation, with the inherently sharp peaks the number of data points can be compromised when coupled to mass spectrometric detection unless using an instrument capable of fast scan speeds. Hence, the separation was initially performed on the 2.5 µm variant to avoid compromising the number of data points, which are required for good peak definition, especially where quantification is required. A gradient separation was optimised to provide suitable resolution of the five ephedrines while maintaining a short analysis time. The final conditions are detailed below with the corresponding chromatographic separation (Table 2.5 and Figure 2.23).

Table 2.5. Final separation conditions.
Optimised conditions for the separation of PPA, cathine, ephedrine, pseudoephedrine and methylephedrine by LC-MS/MS.

Column:	Waters XBridge C_{18} 2.1 x 50 mm, 2.5 µm
Mobile phase A:	10 mM ammonium bicarbonate pH 9.8 in water
Mobile phase B:	10 mM ammonium bicarbonate pH 9.8 in 60 % methanol
Flow rate:	0.5 mL/min
Gradient:	

Time (min)	%A	%B	Curve
0.0	83.3	16.7	-
3.2	58.3	41.7	6
5.2	8.3	91.7	6
6.5	83.3	16.7	1

Run Time:	6.5 minutes
Column Temp:	45 °C
Injection Vol:	10 µL

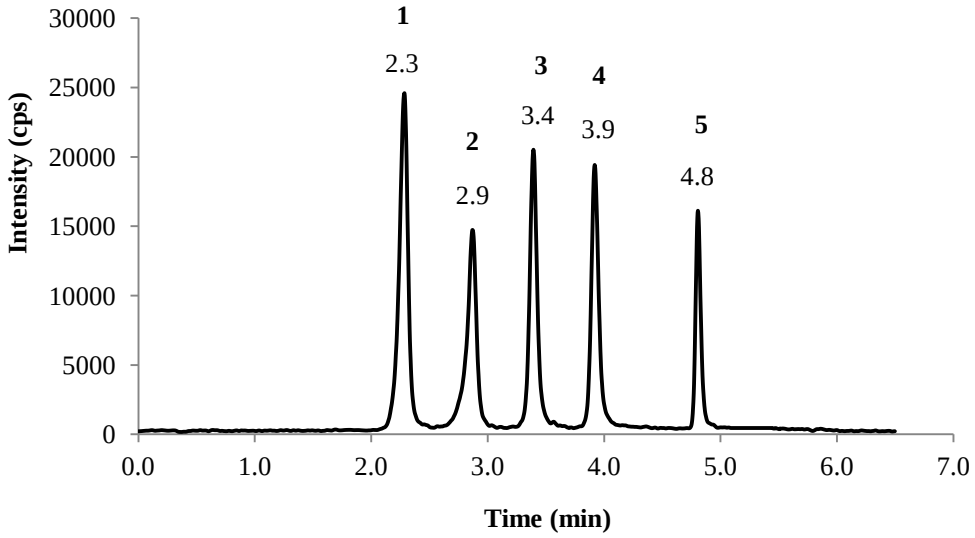


Figure 2.23. Total ion chromatogram (TIC) illustrating the separation of the five ephedrine compounds considered in this study using the final gradient conditions.
PPA (1), cathine (2), ephedrine (3). pseudoephedrine (4) and methylephedrine (5), performed on an XBridge C_{18} 2.5 µm, 50 x 2.1 mm column using the conditions in Table 2.5.

2.5 Conclusions

The results from this work highlight the importance of mobile phase pH for the analysis of basic compounds, where the best chromatographic performance is achieved under high pH conditions to suppress protonation. The difference in performance when operating at elevated pH and temperature can be attributed to a reduction in analyte protonation, encouraging hydrophobic retention and reduced detrimental secondary interactions and peak tailing.

Enhanced peak shape and retentivity permit good chromatographic resolution of the diastereoisomers at pH 9.8, without the need for undesirable additives or the need for column regeneration. Even though this is very close to the pK_a of the analytes presented here, this value was shown to provide reproducible retention times and provide an adequate peak shape while preserving the life of the column, which would be shortened at higher pH values, especially with an operating temperature of 45 °C. Finally, in gradient elution, the five ephedrines are separated with good resolution in a run time of 6.5 minutes.

CHAPTER 3

QUANTITATIVE ANALYSIS OF BASIC COMPOUNDS BY HIGH pH REVERSED-PHASE LC-MS/MS; APPLICATION TO EPHEDRINES IN DOPING CONTROL ANALYSIS

3.1 Introduction

Chapter 2 investigated the effects of manipulating mobile phase pH and detailed the benefits of exploiting high pH eluent conditions for the chromatographic separation of basic analytes by reversed-phase liquid chromatography (RPLC). Although developed using UV detection, the method for separating the hydrophilic basic ephedrine compounds was centred on the use of a volatile buffer to enable MS operation. The optimised separation, utilising an ammonium bicarbonate (10 mM) eluent buffered to pH 9.8 with methanol using gradient chromatography, resulted in complete resolution of the five structurally related ephedrine compounds (phenylpropanolamine (PPA), cathine, ephedrine, pseudoephedrine and methylephedrine) with suitable retention and peak shape. With the inclusion of pseudoephedrine in the Prohibited List, previously not restricted by the World Anti-Doping Agency (WADA) until 2010, a new validated confirmation method for identification and quantification was necessary [123]. Therefore, the aim in this chapter is to couple the LC separation with MS detection and validate the method as a confirmatory assay for the identification and quantification of ephedrines in doping control analysis.

In addition to the benefits of using high pH in achieving enhanced retention, resolution and peak shape, the improved sample loading capacity compared with conventional acidic eluents is of particular importance in the current application. Considering the different threshold concentrations established by WADA, a method to analyse ephedrine and pseudoephedrine, together with their respective metabolites – PPA and cathine – requires a large dynamic range for reliable quantification [90]. With the large sample concentrations typically experienced with these compounds, a

method that can accommodate large sample masses is advantageous and also permits a single simultaneous sample preparation procedure without the need to tailor a higher dilution for pseudoephedrine, with such a high threshold concentration (150 µg/mL).

Electrospray ionisation (ESI) remains the most popular mode of ionisation for the detection of hydrophilic compounds analysed by LC. However, when interfacing LC with MS detection, conditions optimal for chromatographic performance are often not the most favourable for ionisation efficiency. For example, often the best ESI sensitivity is achieved when the analyte is already ionised in solution by using an acidic mobile phase for basic analytes but, as we have seen, this is not conducive to optimal LC performance, and hence a compromise must be made between the two. Although conventional wisdom tells us that the ionisation of basic analytes would be suppressed in a basic environment, it is possible for basic compounds to be ionised with positive ESI in a basic environment; as can acidic compounds in an acidic environment [124-128]. In fact, in some cases, previous studies demonstrate even higher sensitivity for basic compounds in high pH eluents [82, 110, 129]. This phenomenon, which has been referred to as "wrong-way-round ionisation," does not support the ion evaporation theory and suggests that alternative mechanisms of ionisation are dominant [108]. Alternative ionisation mechanisms which have been invoked include charge transfer from protons present in solution to neutral analyte molecules, or via redox reactions at the liquid-gas interface of the droplet [80, 109, 130-132]. This theory is supported by a study of mobile phase additives by Mallet *et al.* [98], where an increase in reagent ions, by increasing the concentration of ammonium hydroxide, elicits an increase in response for basic analytes. This

contradicts the ion evaporation theory which is subject to ion suppression as the concentration of mobile phase additives is increased [98]. An alternative theory, proposed to explain the increase in response of basic analytes under high pH conditions, is that as the chromatographic retention of the unprotonated molecules increases, they therefore elute later in a more organic-rich eluent, thus aiding more efficient ionisation. However, a more recent study confounds this hypothesis, where certain neutral analytes do not demonstrate any change in retention with increasing mobile phase pH, yet still exhibit an increase in ESI response [110].

Although such improvements in sensitivity have been noted with high pH eluents in positive mode ESI, there is limited knowledge of how pH affects signal stability and robustness. During this study, initial experiments showed that interfacing with ESI did not give adequate reproducibility for the quantification of ephedrines in urine over the desired linear dynamic range. Moreover, matrix interferences are a concern, particularly for the direct sample dilution and injection approach adopted herein. Alternative ionisation techniques, such as atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI), are inherently less affected by interference from co-eluting matrix components compared with ESI [86, 133-136]. For this reason, positive APCI is investigated as a comparative mode of ionisation to determine the most stable and reproducible response for the quantification of basic analytes in diluted urine.

In this chapter, particular focus is applied to understanding the effects of mobile phase pH with ESI, which may be detrimental to reliable quantification by LC-MS, by reducing sensitivity and increasing variability. One of the objectives was to build

a better understanding of the effects of mobile phase composition, pH and additives on ESI response and spray stability. A comparison is made between acidic and basic LC eluents to determine the effects on positive ESI with basic analytes. Since the focus here is to investigate the effect of mobile phase composition on ESI sensitivity and stability only, post-column infusions and flow injections are performed to eliminate any variations caused by chromatography. This approach allows a direct comparison of the effects of mobile phase additives and pH on ESI, with a controlled amount of organic component in the eluent and no contribution from retention or peak shape differences. In this investigation, three of the ephedrines (PPA, ephedrine and methylephedrine) were studied, together with salbutamol, clenbuterol and formoterol (

Figure 3.1), chosen because of their importance in doping control where sensitivity and accuracy are paramount for their low level quantification.

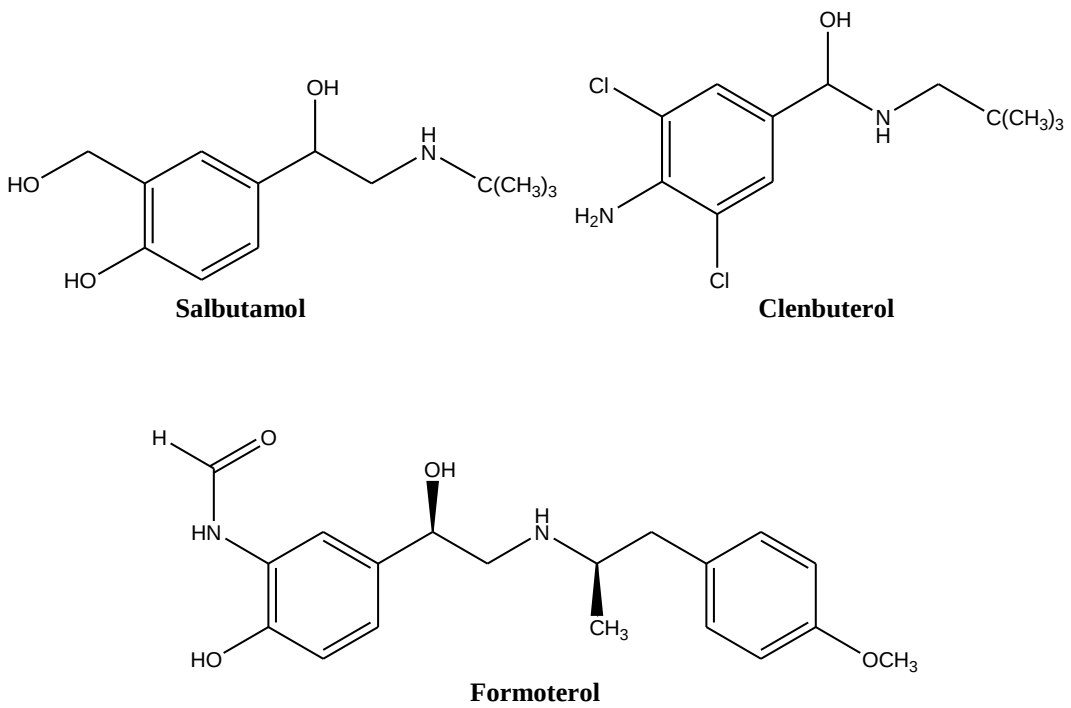


Figure 3.1. Chemical structures of salbutamol, clenbuterol and formoterol.

These compounds included in the study of different solvent systems on ESI sensitivity and stability.

Lastly, a preliminary validation for cathine, ephedrine, pseudoephedrine and methylephedrine is performed on the optimised method for linearity, selectivity, accuracy, precision, carryover and ion suppression due to matrix interferences. The validated LC-MS/MS method is then applied to the measurement of the concentration of ephedrines in two real urine samples and compared with the previously used GC-MS method.

3.2 Experimental

3.2.1 Materials

Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium hydroxide solution (35 % w/w), ammonium formate and ammonium bicarbonate were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99-100 %) was purchased from VWR (Leicestershire, UK). Norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine and clenbuterol, as hydrochloride salts, and salbutamol hemisulfate and formoterol fumarate dihydrate were purchased from Sigma (Poole, UK). Norephedrine-d_3 (used as internal standard) was purchased as a free base (1 mg/mL in methanol) from LGC Standards (Teddington, UK). Water was purified by an ultra-pure water system (Elga, UK).

3.2.2 Solutions

3.2.2.1 Mobile phase

A stock solution of ammonium bicarbonate buffer was prepared at 25 mM in purified water and adjusted to pH 9.8 with ammonium hydroxide solution (35 % w/w) for the preparation of the final mobile phase, which consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60 % methanol (B). For the preparation of 1 L of mobile phase A, 400 mL of the stock buffer solution was added to 600 mL of water in order to achieve a 10 mM buffer solution. For the preparation of mobile phase B, 200 mL of the stock buffer was added to 300 mL of methanol.

3.2.2.2 Samples

Stock solutions were prepared at a concentration of 1 mg/mL for norpseudoephedrine, ephedrine and methylephedrine and 10 mg/mL for pseudoephedrine in methanol and stored at -20 °C. A stock solution of norephedrine-d_3, used as an internal standard (IS), was prepared at 10 µg/mL in methanol. Standard working solutions were prepared by diluting stock solutions with water.

A two-step dilution of urine samples was performed before injection: firstly a 45-fold dilution with water was performed, after which samples were vortexed and centrifuged at 76.7 *g*. Aliquots (200 µL) were then mixed with an equal volume of IS solution (norephedrine-d_3, 500 ng/mL diluted with water). Vials were vortexed before being placed in the autosampler.

3.2.3 LC conditions

Separations were carried out on an Acquity UPLC® system (Waters, Milford, MA, USA) with an XBridge 2.5 µm C_{18} 2.1 x 50 mm column provided with a 0.2 µm in-line filter. The run time was 6.5 minutes including re-equilibration time. The mobile phase consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60 % methanol (B). The flow rate was 500 µL/min and column temperature set at 45 °C. The weak and strong needle wash lines of the Acquity UPLC® system were placed in 90:10 H_2O/CH_3OH 0.2 % formic acid and 10:90 H_2O/CH_3OH 0.2 % formic acid, respectively. The injection volume was 10 µL and was performed in the partial loop with needle overfill mode using a 20 µL sample loop. The gradient conditions started at 16.7 % B, increasing to 41.7 % over 3.2 minutes and to 91.7 % at 5.2 minutes, returning to 16.7 % for a 1.3 minute

re-equilibration. Analyst® 1.4.2 software from Applied Biosystems was used to control the Acquity UPLC®.

3.2.4 Mass spectrometry

Analyte detection was performed using an API 3200 triple quadrupole tandem mass spectrometer (Applied Biosystems) equipped with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) sources in positive ion mode. The MS was operated in selected reaction monitoring (SRM) mode with the inlet conditions optimised for ESI and APCI. The following optimised source conditions were selected for ESI: capillary voltage 5.5 kV; temperature 450 °C; curtain gas (nitrogen) 10 psi; nebuliser gas (nitrogen) 45 psi and auxiliary gas (nitrogen) 80 psi. The conditions selected for APCI were: nebuliser current 3 µA; temperature 400 °C; curtain gas (nitrogen) 10 psi and auxiliary gas (nitrogen) 45 psi. Data acquisition was divided into four segments based on the expected retention times, with a dwell time of 100 msec for each transition giving sufficient data point sampling and sensitivity. Three ion transitions and the precursor ion are all monitored for each analyte in order to satisfy the WADA requirements for identification [137]. Acquisition segments, selected reaction monitoring (SRM) transitions, dwell times and MS parameters are detailed in Table 3.1.

As a comparative instrument, an Agilent 6410 LC-MS/MS system was used. The same LC conditions were applied, but the MS parameters were optimised for this instrument. The same SRM transitions were acquired using ESI in the positive mode with the following conditions: capillary voltage 1.5 kV, temperature 350 °C, nebuliser gas (nitrogen) 45 psi, drying gas (nitrogen) flow 13 L/h.

Table 3.1. Acquisition segments, SRM transitions and MS parameters.
Declustering potential (DP), entrance potential (EP), collision cell exit potential (CXP) and collision energy (CE) for the ephedrines considered in the study. The dwell time of each ion transition was 100 msec.

Compound	Retention window (min)	Ions monitored (m/z)	DP (V)	EP (V)	CXP (V)	CE (V)
Cathine	**2.3-2.9**	152.1*	27	5.0	-	-
		134.1**			9	15
		117.2			9	24
		115.2			9	32
Ephedrine	**2.9-4.4**	166.1*	30	6.5	-	-
		148.3**			12	16
		133.0			9	30
		117.1			11	26
Pseudoephedrine	**2.9-4.4**	166.1*	30	6.5	-	-
		148.2**			12	16
		133.1			9	30
		117.1			11	26
Methylephedrine	**4.4-6.5**	180.2*	35	6.0	-	-
		162.2			14	17
		147.1**			12	27
		135.2			10	21
		117.2			10	26
IS (norpehedrine - d_3)	**0-2.3**	155.1*	30	5.0	12	12
		137.2**				

*Precursor ion used, **Transitions used for quantification.

3.2.5 Calibration

A six-point calibration curve including 50-200 % of the threshold concentrations was constructed by spiking blank human urine at 1, 2.5, 5, 10, 15, 20 µg/mL for cathine, 2.5, 5, 10, 20, 30, 40 µg/mL for ephedrine and methylephedrine and 25, 50, 100, 200, 300, 400 µg/mL for pseudoephedrine (Table 3.2). Quality control (QC) samples were prepared at concentrations equal to the WADA threshold levels (cathine 5 µg/mL, ephedrine 10 µg/mL, pseudoephedrine 150 µg/mL and methylephedrine 10 µg/mL).

Table 3.2. The concentrations of the calibrants and QC (µg/mL) prepared in blank urine. (Eph = ephedrine, Peph = pseudoephedrine, Meph = methylephedrine).

Calibrant	Volume (µL)				Final volume (mL)	Concentration (µg/mL)			
	Cathine	Eph	Peph	Meph		Cathine	Eph	Peph	Meph
1	0	0	0	0	10	0	0	0	0
2	200	50	50	50	20	1	2.5	25	2.5
3	250	50	50	50	10	2.5	5	50	5
4	500	100	100	100	10	5	10	150	10
5	500	100	100	100	5	10	20	200	20
6	750	150	150	150	5	15	30	300	30
7	1000	200	200	200	5	20	40	400	40
QC	500	100	100	100	10	5	10	150	10

3.2.6 Validation

The method was validated for linearity over the dynamic range, selectivity, accuracy, precision, carryover, and ion suppression due to matrix interferences. Ten blank urine samples obtained from different volunteers were analysed as described above to ensure selectivity of the method. Linearity was determined with the six-point calibration and repeatability was assessed by analysing six replicates of a urine sample spiked with each analyte at the threshold concentration. Accuracy and between-assay precision were determined by analysing three replicates of the three different spiked urines on different days.

3.2.7 Post-column infusion

Where used, the post-column acidification reagent was 10 % formic acid in purified water, infused at 20 µL/min between the LC outlet and MS inlet using a T-connection by means of a JASCO PU-1585 HPLC pump (JASCO, Tokyo, Japan). For infusion at 20 µL/min with a mobile phase flow rate of 500 µL/min, a 10 % formic acid solution at was necessary to achieve an apparent eluent pH < 7, ensuring

full analyte ionisation in solution. For comparison, analysis with no post-column acidification was performed with an infusion of purified water at the same flow rate to compensate for any dilution effects.

3.2.8 Flow injection

Phenylpropanolamine (PPA), ephedrine, methylephedrine, salbutamol, clenbuterol and formoterol were used as basic test probes to evaluate ESI sensitivity and stability with different mobile phase systems. Flow injection was performed using a zero-volume connector to eliminate any variations from chromatographic contribution. An Acquity UPLC® system was used with a Xevo QTOF (Waters, Manchester, UK) mass detector. The amount of organic content was kept constant, using 20 % acetonitrile or methanol with the particular aqueous mobile phase investigated; 0.1 % formic acid in water, 0.1 % ammonium hydroxide in water, 10 mM ammonium formate pH 3 or 10 mM ammonium formate pH 10. The QTOF mass spectrometer was operated in full scan mode and the source parameters tuned for the protonated molecular ion for each analyte by infusion under each set of conditions. The optimised conditions for each analyte are detailed in Table 3.3. A flow rate of 0.5 mL/min was used throughout the investigation. Prior to injection, samples were diluted with the appropriate mobile phase to 500 ng/mL and 2 µL injections were made.

Table 3.3. Optimised MS source parameters.
Cone voltage (CV), sample cone (SC), extraction cone (EC), source temperature (ST), desolvation temperature (DT), cone gas (CG) and desolvation gas (DG), for each compound under each set of mobile phase conditions.

	PPA	Ephedrine	Methylephedrine	Salbutamol	Clenbuterol	Formoterol
Cone voltage (kV)	0.5	0.5	0.5	0.5	0.5	0.5
Sample cone (V)	15	15	15	20	20	25
Extraction cone (V)	2	2	2	4	2	2
Source temp. (°C)	120	120	120	120	120	120
Desolvation temp. (°C)	600	600	600	600	600	600
Cone gas (L/h)	10	10	10	10	10	10
Desolvation gas (L/h)	800	800	800	800	800	800

3.3 Results

3.3.1 Interfacing with Mass Spectrometry (LC-MS/MS)

Since MS detection is necessary for analyte identification, a compatible eluent composition was carefully selected to avoid undue compromise of the LC separation. Additives and high buffer concentrations, although frequently used to improve chromatographic separations, are incompatible with MS since they often cause ion suppression. Ammonium bicarbonate, at a concentration of 10 mM, was selected for its buffering capacity at high pH and volatility, hence amenable to MS detection. The buffer component was also added to the organic solvent to maintain a uniform concentration throughout the chromatographic gradient. Gradient conditions, temperature and flow rate were optimised in order to obtain the best separation within a maximum analysis time of 10 minutes.

Figure 3.2 illustrates the final chromatographic separation of the four ephedrines of interest at the WADA threshold levels (cathine 5 µg/mL, ephedrine 10 µg/mL, pseudoephedrine 150 µg/mL and methylephedrine 10 µg/mL) in spiked urine.

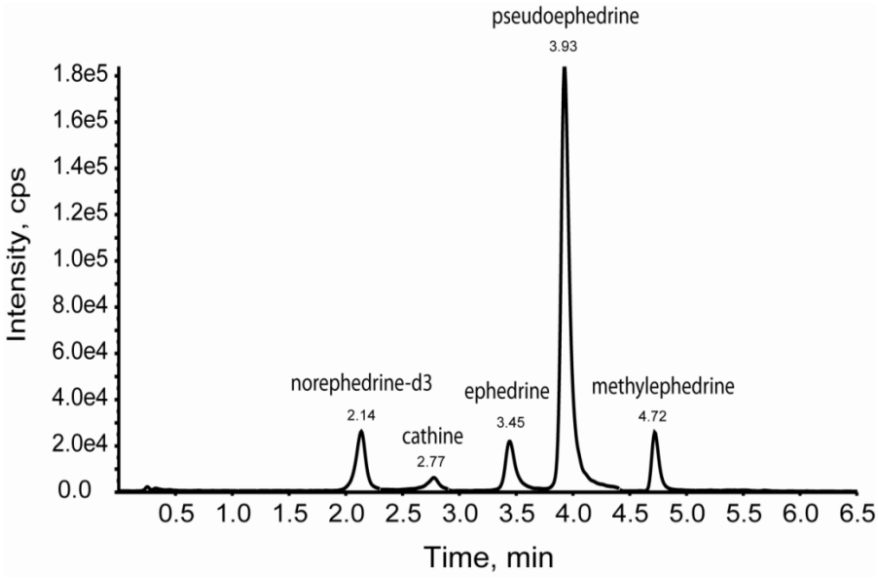


Figure 3.2. Total ion chromatogram (TIC) illustrating the separation of cathine, ephedrine, pseudoephedrine and methylephedrine.
Analytes spiked at the WADA threshold concentrations in urine (cathine 5 µg/mL, ephedrine 10 µg/mL, pseudoephedrine 150 µg/mL, methylephedrine 10 µg/mL) and with the IS norephedrine-d_3 (250 µg/mL). Separation performed on an XBridge C_{18} 2.5 µm, 50 x 2.1 mm column at 0.5 mL/min.

Since the ephedrine compounds considered in this study have similar structures, they share common fragmentation patterns (Figure 3.3), hence the requirement for chromatographic separation prior to detection. However, despite the chromatographic optimisation in order to achieve this being performed with a volatile MS compatible buffer system, preliminary experiments with positive ESI indicated poor signal stability and repeatability. Various stages of the analysis were investigated to determine the cause of these poor results. Overloading of the column or detector and inaccuracy or losses in the sample preparation were all investigated as potential sources of problems.

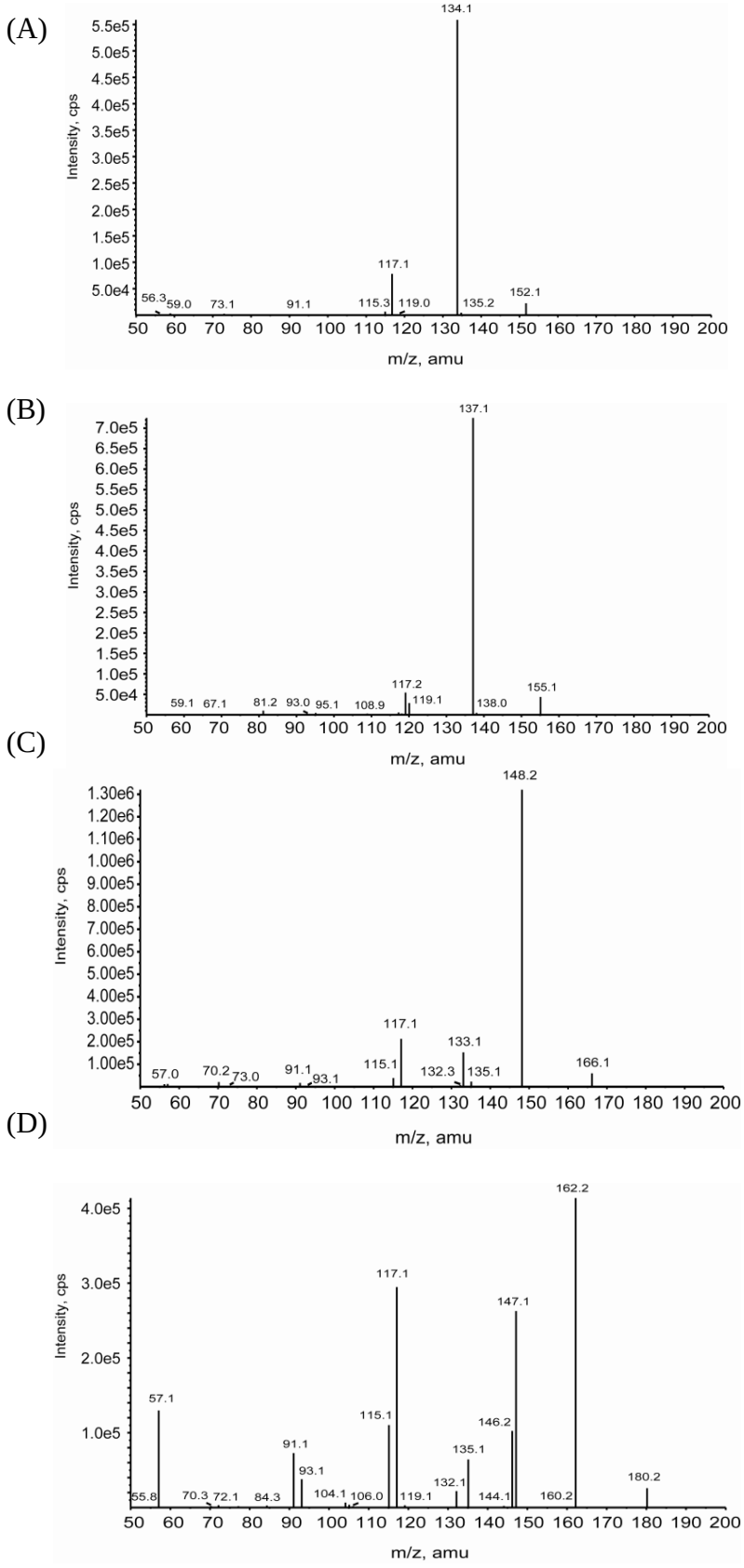


Figure 3.3. ESI product scan spectra of cathine (A), norephedrine-d_3 (B), ephedrine and pseudoephedrine (C) and methylephedrine (D).

The precursor ions highlighted in Table 3.1. were used.

3.3.1.1 Sample preparation

The influence of sample pre-treatment is paramount in the speed and simplicity of analysis, as well as adding potential sources of error. The sensitivity of LC-MS provides the possibility of directly diluting and injecting the sample, without the need for pre-concentration and derivatisation, particularly considering the high concentrations being analysed here. However, as explained in the previous chapter, the effect of large sample masses injected on column can have severe negative implications for the chromatographic performance, even though this is less apparent with basic analytes in a basic eluent. In an overload situation, peaks exhibit large tailing factors which confound accurate peak integration. With such high concentrations of pseudoephedrine being injected on column, various dilution factors were applied to ensure column overload was not contributing to poor quantification. The dilution factor was determined in order to simultaneously identify the lowest abundant ion for cathine at the lowest calibration concentration (1 µg/mL) while not overloading the column or saturating the detector with the highest concentration of pseudoephedrine (400 µg/mL). A 90-fold dilution was determined suitable for satisfying these criteria.

The dilution approach adopted comprises a two stage dilution; firstly a 1:45 dilution with water, followed by a 1:1 dilution with norephedrine-d_3 internal standard (500 ng/mL in mobile phase). Dilution was performed with purified water instead of mobile phase to eliminate possible evaporation of the methanol-containing diluent during the dilution steps. Using water as the diluent did not have any negative effect on chromatographic performance since the starting composition is largely aqueous

and water acts as an even weaker eluent. Following the first stage of the dilution, the samples were centrifuged at 76.7 *g* to remove any particulate matter from the urine samples. The dilution procedure involves the use of Gilson and Eppendorf multi-dispensing pipettes, and these were eliminated as a source of error by taking 10 measurements with each and calculating % RSD values, found to be < 0.76 %.

3.3.1.2 Instrumentation

Having not been able to determine any sources of error during the sample preparation procedure, the next approach was to investigate the instrumentation. The same samples were run on an alternative LC-MS/MS system with a different source design to determine whether the instrumentation was contributing to any error. However, the same problem was replicated on both instruments, omitting the possibility of an instrumentation issue.

3.3.1.3 Comparison of ESI and APCI

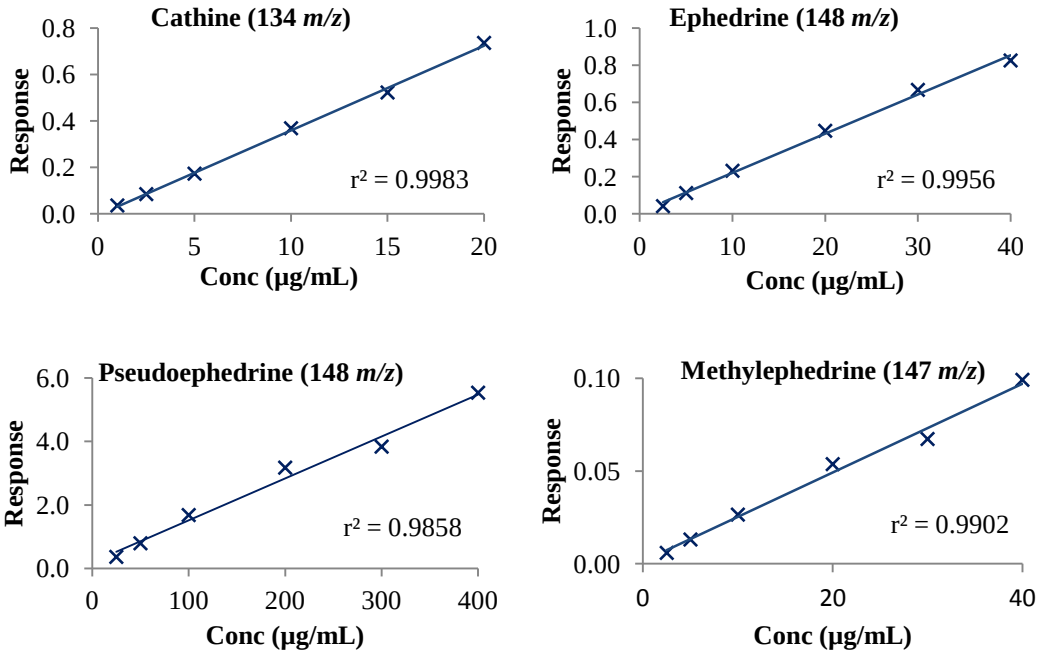
Since the use of APCI is more appropriate for the analysis of relatively semi/non-polar compounds, and in this case the ephedrine compounds are entering the source in a largely deprotonated state, this mode of ionisation was investigated as a comparator. It is also well known that APCI is less susceptible to matrix interferences, which is particularly important in this instance whereby urine is diluted and injected directly without any extensive sample clean-up procedures.

A 6-point calibration was performed ranging from 50-200 % of the threshold concentrations using ESI and APCI in positive ion mode. An additional change that

was made was the quantification ion for pseudoephedrine. Considering the large threshold limit for pseudoephedrine (150 µg/mL), which expands the dynamic range of the assay, the resulting signal is tenfold greater for pseudoephedrine than the other compounds. Quantification of pseudoephedrine using the ^{13}C isotope was therefore investigated to reduce the mass spectrometric signal to the same magnitude as the other ephedrine compounds. This approach was effective in reducing the signal by a factor of 10 compared with the ^{12}C isotope, although it did not have any significant influence on linearity.

For both ESI and APCI, linearity plots were generated from the most abundant ion transition of each compound (Figure 3.4). ESI signals were determined to be less stable, being either non-linear or having significantly reduced linear ranges compared with APCI. APCI yielded a linear and stable signal for each analyte over the wide calibration range, with a correlation coefficient (r^2) greater than 0.995 for each ion transition.

(A) ESI



(B) APCI

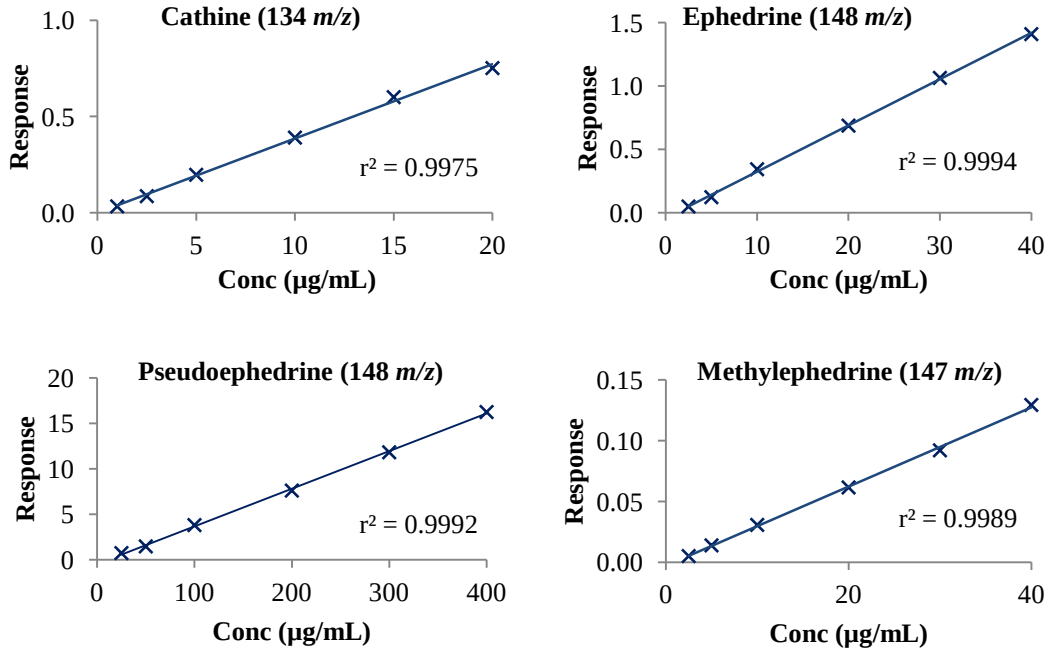


Figure 3.4. Linearity plots for the ephedrine compounds in urine using ESI (A) and APCI (B). Response calculated using analyte peak area divided by internal standard peak area.

The variation in response with ESI compared with APCI is illustrated by overlaid chromatograms from six repeat injections of pseudoephedrine at 150 µg/mL (Figure 3.5).

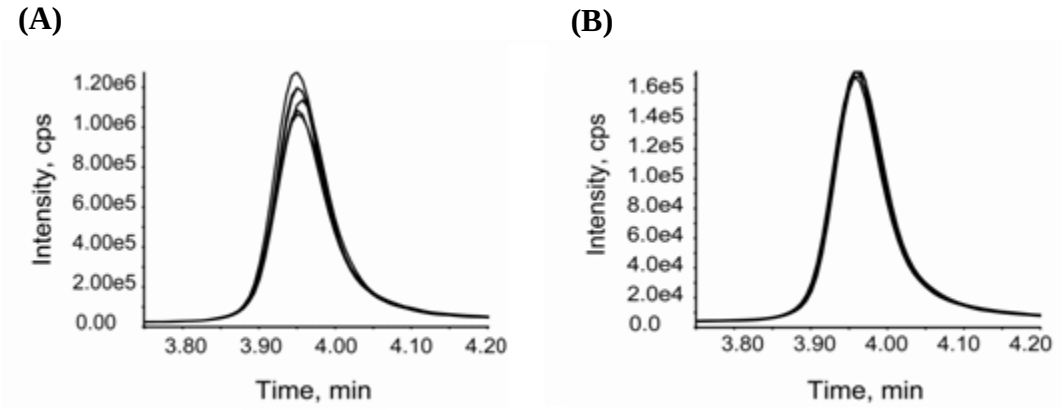


Figure 3.5. Overlay of pseudoephedrine from replicate injections using ESI (A) and APCI (B). Urine sample spiked at the threshold level (150 µg/mL), (n = 6).

Although first experienced with standard solutions without the presence of matrix components, this difference was particularly noticeable for pseudoephedrine in spiked urine, with r^2 values of 0.9858 and 0.9992 generated with ESI and APCI respectively. The errors between the determined and the actual concentrations were outside of our acceptable limit with ESI, whereas APCI illustrated acceptable errors, with values for pseudoephedrine of 49.3 % (n = 6) compared with 15.4 % (n = 6) at the lowest calibration point (Table 3.4).

Table 3.4. Comparison of ESI and APCI for the quantification of pseudoephedrine. Dynamic range 25-400 µg/mL in urine using the 166 →148 m/z transition.

	Conc. (µg/mL)	Slope	Intercept	r^2	Calculated conc. (µg/mL)	Error (%)
ESI	25	0.0132	0.1926	0.9858	12.7	-49.3%
	50				45.1	-9.8%
	100				112	12.4%
	200				225	12.7%
	300				275	-8.2%
	400				404	1.0%
APCI	25	0.0413	-0.4604	0.9992	28.8	15.4%
	50				46.7	-6.6%
	100				103	3.3%
	200				195	-2.4%
	300				297	-1.0%
	400				404	1.0%

The greater variability with ESI seen with samples in matrix compared with standard solutions suggests some interference from matrix components. It was initially proposed that, in this case, the direct dilution and injection approach was not amenable to ESI, whereas APCI offered an alternative in eliminating matrix effects without requiring an extraction procedure. Therefore, matrix effects were quantified using both ESI and APCI to compare the contribution from endogenous interferences. This was evaluated using 10 different blank urine samples spiked at threshold concentration levels with each analyte. Matrix effect was calculated by comparing the response of each analyte spiked in urine to standards prepared in mobile phase. The matrix effect value of < 100 % indicates ion suppression and > 100 % indicates ion enhancement. However, the results indicated that both ESI and APCI are affected to a similar extent, with ion suppression from possible matrix interference shown to be negligible for both methods of ionisation (Figure 3.6).

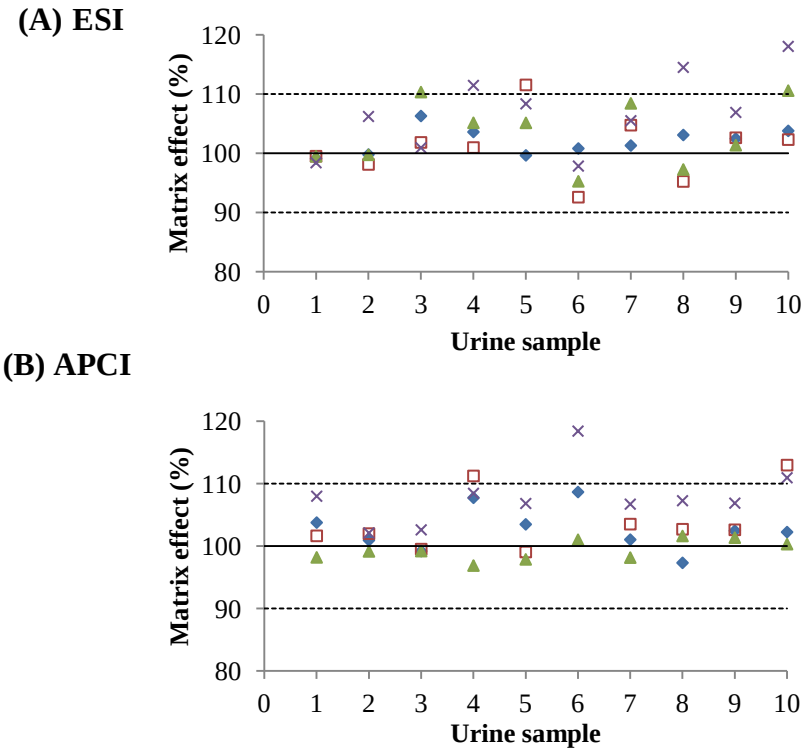


Figure 3.6. Matrix effects evaluated in 10 different spiked urine samples using ESI (A) and APCI (B).
Cathine (♦), ephedrine (□), pseudoephedrine (▲) and methylephedrine (x).

3.3.1.4 Post-column infusion

Several studies report the effect of eluent composition on ESI signal intensity, however there is limited information available concerning the effect on ESI signal stability. The poor stability noted here with basic mobile phase and ESI instigated an evaluation into the effect of mobile phase pH on the signal stability of ephedrines with ESI. In order to determine whether the eluent pH of the solution entering the ion source affects the stability of analyte ionisation with ESI, post-column infusion to acidify the basic eluent was also investigated. In order to acidify the high pH mobile phase, post-column acidification was performed to investigate the effects of pH on signal intensity and stability. Infusion of a 10 % formic acid at 20 µL/min was required to render the eluent at pH < 7. The results acquired with and without post-

column acidification are presented in Figure 3.7. These indicate that ESI response is significantly affected by pH, with a higher response obtained for each analyte under acidic eluent conditions. This is supported by traditional theories that analytes will be detected more easily if they are ionised in solution prior to MS detection. However, more recent reports have shown that higher sensitivities can be achieved for basic analytes using basic eluents with ESI in the positive mode [82, 110, 129]. This phenomenon is likely to be analyte specific, and one theory suggests that enhanced response under high pH conditions is a result of the longer retention of unprotonated analytes with high pK_a values which therefore elute later in a higher organic content. Whereas more recent studies illustrate that, even if retention is unaffected by mobile phase pH, sensitivity is still improved under basic conditions [110]. However, the primary concern in this study is the robustness of the ionisation technique, not necessarily the intensity of the signal, since sensitivity is not a concern. However, although the variation in signal was found to be slightly higher with the basic eluent, this was not found to be significant ($p > 0.05$) (Figure 3.7).

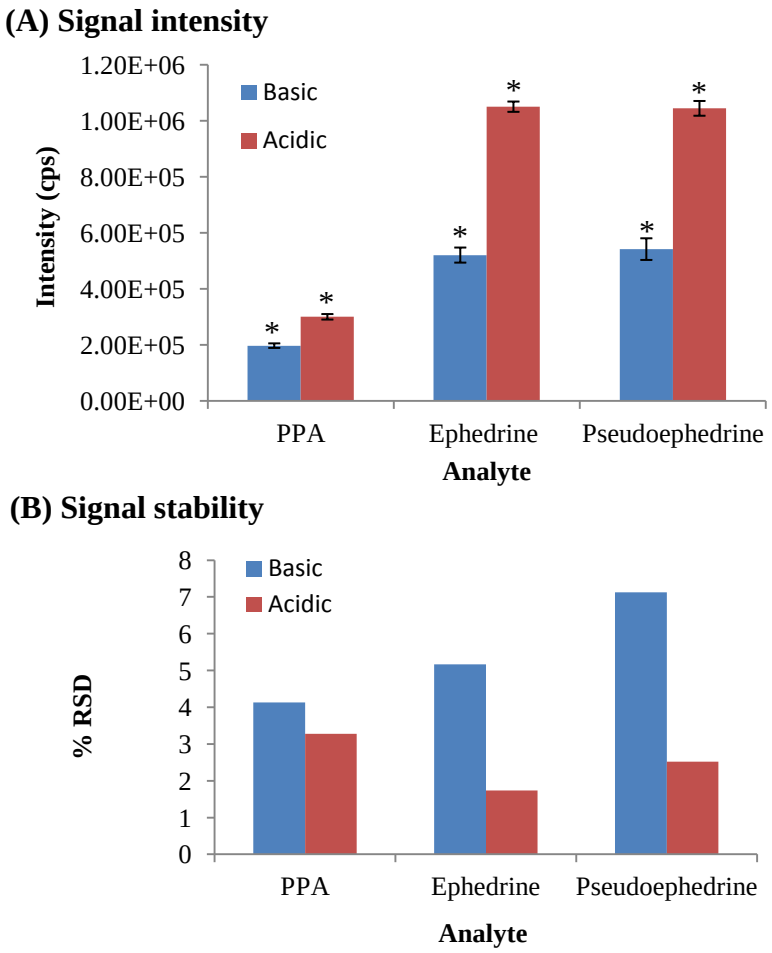


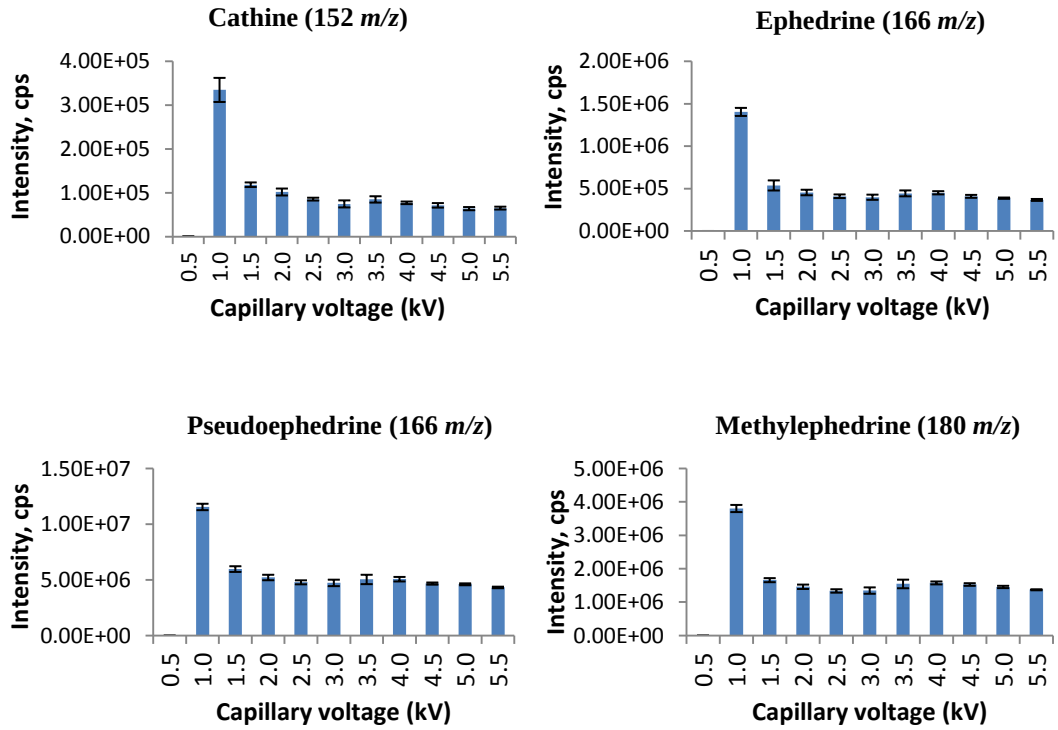
Figure 3.7. Effect of post column acidification (PCA) on signal and intensity (A) and stability (B) with ESI.
Analytes considered were phenylpropanolamine (PPA), ephedrine and pseudoephedrine (n=10), * = statistically significant ($p < 0.05$).

3.3.1.5 Effect of source conditions on ESI response with basic eluent

The optimisation of ESI source parameters, including capillary voltage, cone voltage and gas and temperature settings, is largely influenced by the mobile phase used and the flow rate of the eluent. Following the optimisation of MS conditions which are analyte specific, including collision energy, which is typically performed by infusion of the analyte, infusion with mobile phase flow should be performed to determine the best source conditions for a particular application. For an assay containing a variety

of compounds with different properties or eluting in different amounts of organic content, conditions must be generic and often a compromise between analytes. The importance of capillary voltage on ESI spray is highlighted by the following data under basic conditions. During this study, the effect of capillary voltage on stable spray was investigated by injecting each analyte at voltages ranging from 1.0-5.5 kV, taking average intensities and % RSD values over 10 replicate injections. The results, illustrated for the precursor ions in Figure 3.8 (A) and (B), show an interestingly significant increase in signal intensity at a low capillary voltage of 1.0 kV, yet no significant differences were noted in signal stability. The significant increase in signal at lower capillary voltages with the basic eluent can be explained by the surfactant nature of ammonium bicarbonate, acting to decrease the surface tension of the droplets in the source and therefore making it easier for evaporation to occur, hence increasing ion transfer and detector response (Figure 3.8). In addition to the $[M+H]^+$ ion, the $[M-H_2O]^+$ ion for each analyte was monitored to determine whether dehydration was related to capillary voltage, affecting the signal intensity of the $[M+H]^+$ ion monitored. However, this data was similar to the $[M+H]^+$, suggesting that dehydration is not affected by capillary voltage.

(A) Signal intensity



(B) Signal stability

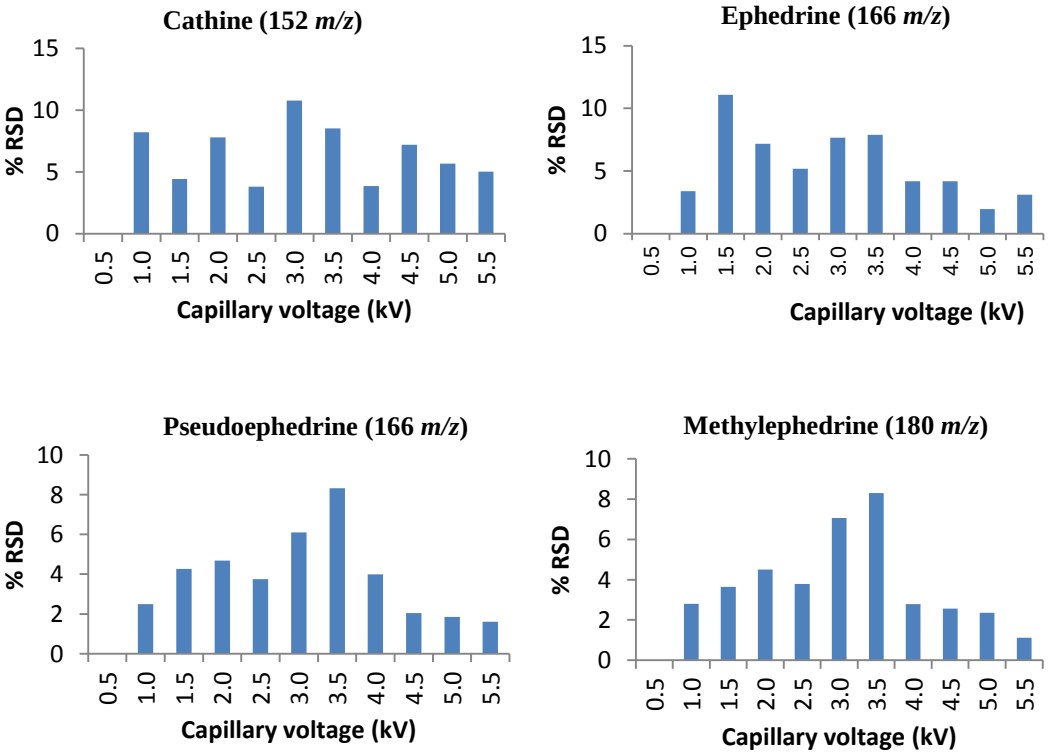


Figure 3.8. Influence of capillary voltage and ESI signal intensity (A) and stability (B). A high pH (pH 10) eluent was used with ESI operated in the positive mode (n = 10).

3.3.1.6 Comparison of mobile phase additives

The importance of optimising ESI source conditions and MS parameters for sensitivity and stability has been previously highlighted. In addition to analyte properties, optimal conditions are dependent on chromatographic conditions, including mobile phase composition, pH, buffer concentration, additives and flow rate. The effect of a variety of viable mobile phases on ESI sensitivity and stability was investigated. In this study, another alternative instrument, equipped with a source design which can accommodate flow rates up to 2.0 mL/min, is used to further evaluate the effect of mobile phase pH and additive on ESI response signal intensity and stability. Additives, including 0.1 % ammonium formate, ammonium formate (10 mM) adjusted to pH 3, 0.1 % ammonia and ammonium bicarbonate (10 mM) adjusted to pH 10, were evaluated with either acetonitrile or methanol as the organic modifier.

Given the different source designs available between different instruments and manufacturers, conditions will vary from instrument to instrument. In order to maximise ESI sensitivity and stability, parameters should be tuned for each analyte under the appropriate mobile phase conditions and flow rate used. Since SRM conditions are traditionally optimised by infusion prior to developing the optimal chromatographic separation, the mobile phase conditions may be quite different from those used in the initial MS optimisation. ESI source conditions were, therefore, optimised for each analyte under each set of conditions with the appropriate mobile phase at a flow rate of 0.5 mL/min (Table 3.3). Again, a low capillary voltage (0.5 kV) typically gave the most intense response with these basic analytes, however

this was not a reflection of the mobile phase used, rather the chemistry of the analyte.

Signal response was determined by peak area, which is the most commonly used measure of signal intensity in quantitative analyses. While signal-to-noise ratio can be a useful measure between different systems, large variations can be experienced, especially in highly sensitive instrumentation where electronic noise is apparent. Ten replicate injections were made of each analyte under each set of conditions and the integrated peak areas averaged for statistical evaluation. Mean peak areas of these 10 replicate injections are presented in Figure 3.9. For all of the analytes studied a greater response is elicited when using a high pH mobile phase compared with an acidic mobile phase, although the degree of the increase is analyte specific. The increase in response with either 0.1 % ammonium hydroxide or 10 mM ammonium bicarbonate pH 10 was as great as three-fold for certain analytes (PPA, salbutamol and formoterol). However, there was no significant difference between the use of a buffered mobile phase or the simple addition of acid or base. Additionally, although there are some minor differences between the organic modifiers used, with a tendency for a slightly increased signal with methanol, this does not contribute significantly to sensitivity for the analytes studied.

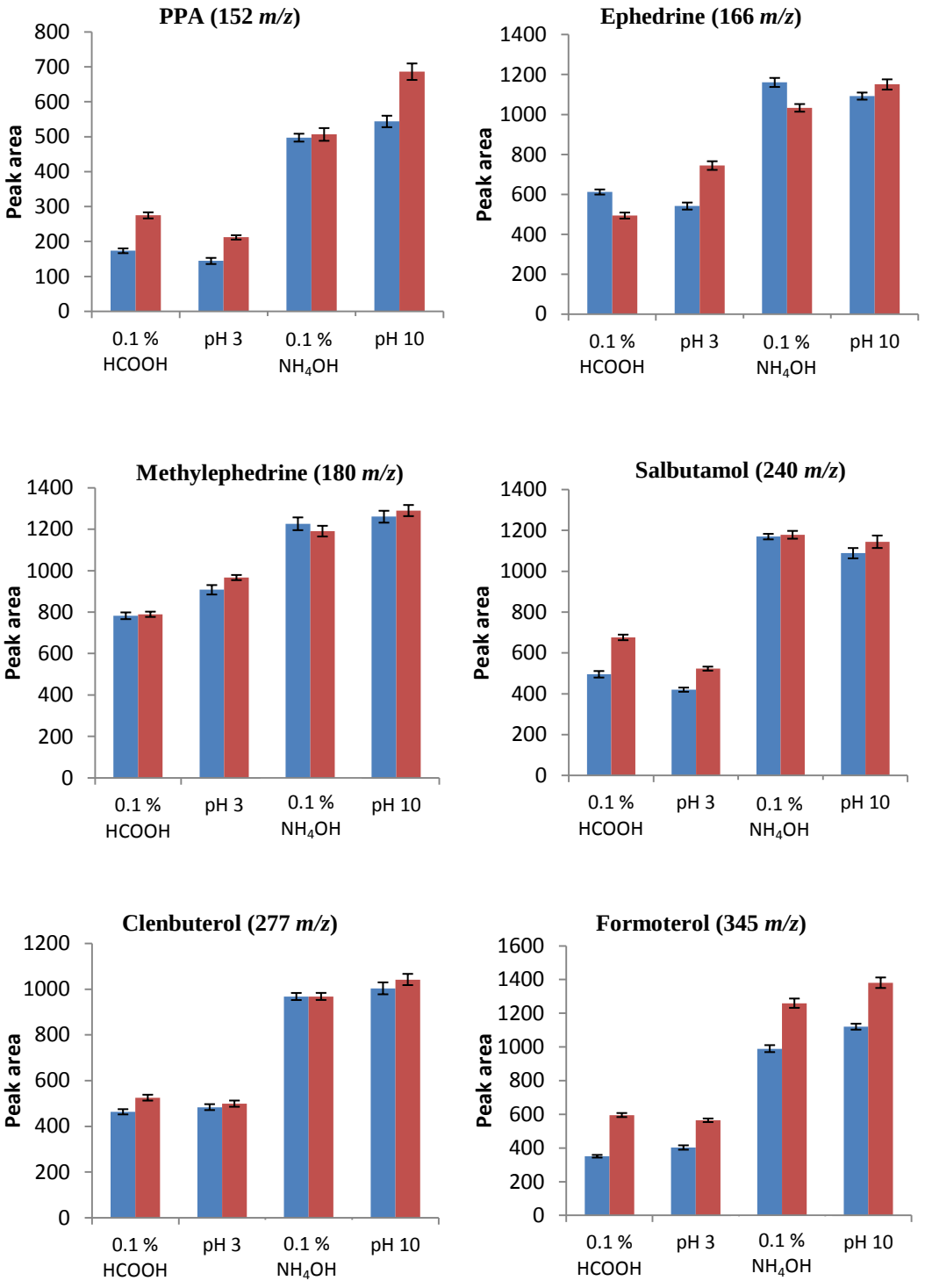


Figure 3.9. Effect of pH and mobile phase additive on peak area.
Flow injection ESI +ve ionisation using CH_3CN (blue) and CH_3OH (red) was employed.

In addition to ESI response, stability was also evaluated for the different solvent systems over 10 replicate injections. The results of the stability experiments are represented as % RSD values in Figure 3.10, which demonstrate that there are no significant differences in ESI spray stability with the different solvent systems studied. The maximum % RSD value found was for PPA using 10 mM ammonium formate pH 3 with acetonitrile at 6.07 % (n = 10). All other values were calculated to be less than 3.94 % RSD (n = 10).

Since chromatographic effects have been eliminated in this study, it can be concluded that the increased response with high pH mobile phases is not a result of elution in a higher organic area of the chromatogram due to enhanced retention, nor due to differences in peak shape. One hypothesised contributing factor is the surfactant behaviour of ammonium hydroxide in the high pH eluents, acting to reduce the surface tension of solvent droplets which aids desolvation and, therefore, more efficient ionisation.

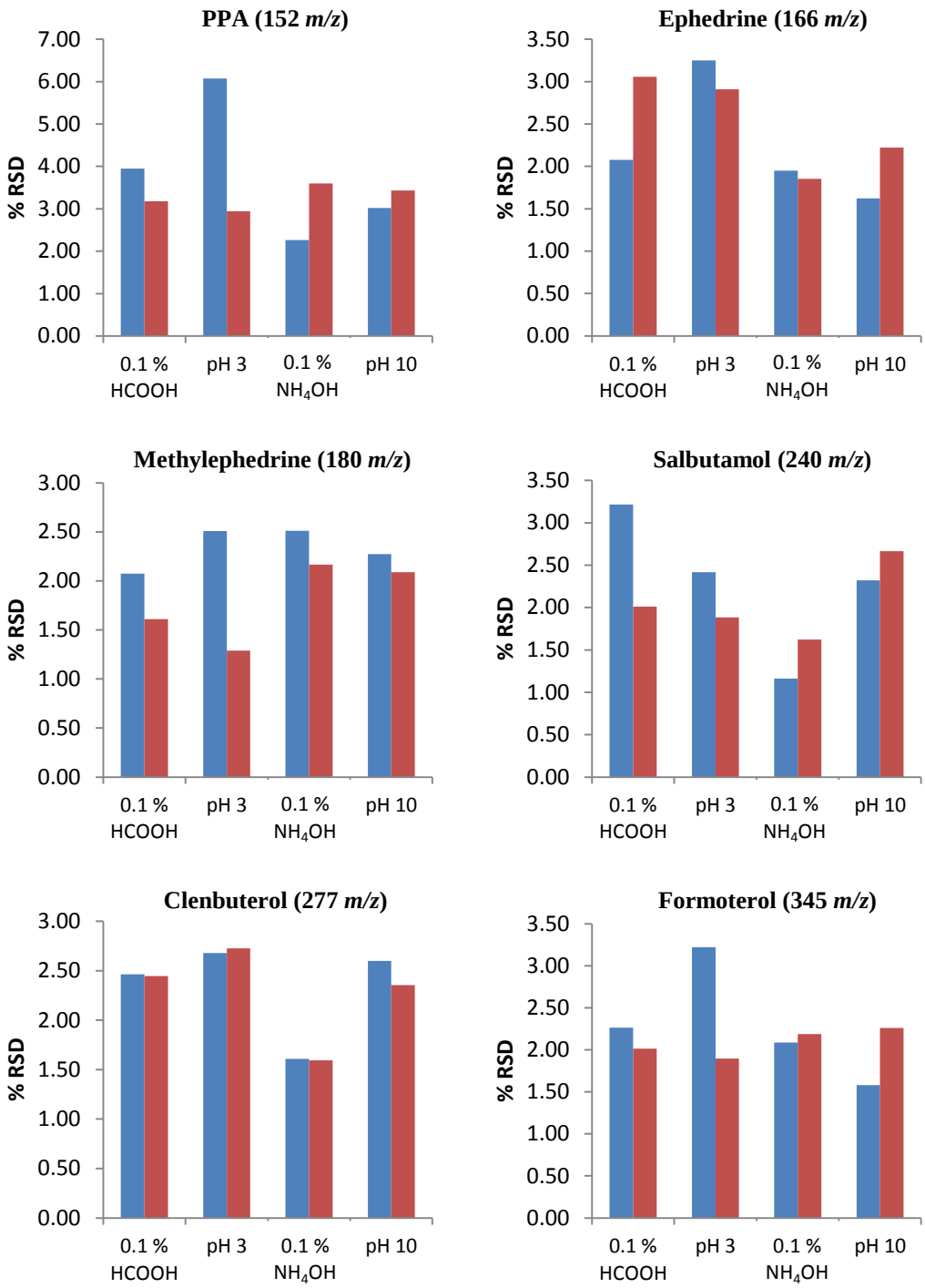


Figure 3.10. Effect of pH and mobile phase additive on peak area stability.
Flow injection ESI +ve ionisation using CH_3CN (blue) and CH_3OH (red) was employed. For % RSD calculations n = 10.

3.3.2 Validation

Since the LC-APCI-MS/MS method showed good linearity over the dynamic range for each analyte, this assay was validated in terms of linearity, selectivity, accuracy and precision, carryover and ion suppression due to matrix effects. Multiple analyses of a quality control (QC) sample at the threshold concentration for each analyte were obtained. Cathine was analysed at 5 µg/mL, ephedrine and methylephedrine were analysed at 10 µg/mL and pseudoephedrine at 150 µg/mL. The QCs were prepared by spiking analytes into blank urine at the same time that the calibrants were prepared. Validation consisted of three preparations on three different days. The concentration of each QC was calculated against a calibration curve (prepared freshly for each batch). The following parameters were used to ensure that adequate accuracy and precision were obtained from the assay:

$$Accuracy = \frac{mean\ concentration}{nominal\ concentration} \times 100\ \%$$

$$Within - assay\ precision = \frac{standard\ deviation\ from\ within\ assay\ means}{average\ of\ within\ assay\ means} \times 100\ \%$$

$$Between - assay\ precision = \frac{standard\ deviation\ from\ between\ assay\ means}{average\ of\ between\ assay\ means} \times 100\ \%$$

3.3.2.1 Linearity

A six-point calibration curve including 50-200 % of the WADA threshold levels has been generated to confirm linearity over the range for quantification. Correlation coefficients (r^2) were greater than 0.9992 (Table 3.5). Errors (calculated as the difference between the determined and actual concentration) ranged between -7.5 and 2.1 % over a concentration range corresponding to 50-200 % of the WADA threshold levels for cathine (5 µg/mL), ephedrine (10 µg/mL), pseudoephedrine (150 µg/mL) and methylephedrine (10 µg/mL).

Table 3.5. Linearity results for calibration of cathine, ephedrine, pseudoephedrine and methylephedrine.

Compound	Ion (m/z)	Range (µg/mL)	Slope	Intercept	r^2	Equation
Cathine	134	1.0-20	0.0445	-0.0045	0.9997	y = 0.0445x - 0.0045
Ephedrine	148	2.5-40	0.0658	-0.0206	0.9992	y = 0.0658x - 0.0206
Pseudoephedrine	148	25-400	0.0583	-0.5223	0.9995	y = 0.0583x - 0.5223
Methylephedrine	147	2.5-40	0.0079	-0.0024	0.9999	y = 0.0079x - 0.0024

3.3.2.2 Selectivity

Selectivity was determined through the analysis of 10 different blank urines with no interference being detected at the expected retention times of the analytes.

3.3.2.3 Accuracy and precision

The method was tested for accuracy and precision through the analysis of three repeats of urine spiked with each analyte at the WADA threshold level performed on three different days. The results for accuracy, reported as % bias between the estimated and actual concentration values, are all less than 10 %, and % RSD values

for within- and between-assay variation are less than 7.54 and 5.77 (n = 3), respectively (Table 3.6).

Table 3.6. Within- and between-assay precision (% RSD) and accuracy (% bias) for the ephedrines in urine at QC concentrations (n = 3).

Compound (µg/mL)		Mean conc (µg/mL)	Accuracy (% bias)	Precision (% RSD)	
				Within-assay	Between-assay
Cathine (5 µg/mL)	**Day 1**	4.98	-0.47	3.22	4.14
	Day 2	4.64	-7.13	7.54	
	Day 3	5.01	0.13	4.20	
Ephedrine (10 µg/mL)	**Day 1**	10.4	3.50	3.51	5.20
	Day 2	9.36	-6.37	2.19	
	Day 3	9.64	-3.60	5.08	
Pseudoephedrine (150 µg/mL)	**Day 1**	155	3.09	1.48	3.65
	Day 2	153	1.90	1.21	
	Day 3	164	9.03	1.65	
Methylephedrine (10 µg/mL)	**Day 1**	11.0	9.50	3.71	5.77
	Day 2	9.89	-1.07	2.14	
	Day 3	9.96	-0.43	4.01	

3.3.2.4 Carryover

Carryover was determined by injecting urine samples spiked with the analytes at a concentration corresponding to five times the WADA threshold, followed by injection of blank mobile phase. No peak was detected in the blank sample.

3.3.2.5 Matrix effects

The importance of ion suppression due to matrix components in the urine is emphasised, since no extraction was performed before injection. This was determined by comparing the responses of 10 different urine samples spiked with

each analyte at the WADA threshold level with a spiked standard at the same concentration prepared in water. For all compounds, the matrix effect was less than 13%.

3.3.3 Application to real samples

The suitability of the method is demonstrated by the analysis of two samples previously determined positive for cathine and ephedrine respectively by using a validated GC-MS method. LC-MS/MS chromatograms are reported in Figure 3.11. The estimated concentrations are 7.56 µg/mL and 283 µg/mL of cathine and pseudoephedrine in sample C, and 48.3 µg/mL ephedrine in sample D. These mean values are similar to those obtained by GC-MS, estimated at 7.85 µg/mL and 44.7 µg/mL for cathine and ephedrine respectively, all measured in triplicate. With the LC-MS/MS method, pseudoephedrine was detected and quantified in sample A, while the corresponding GC-MS data is not available since the substance was not prohibited in sport when the original analysis was performed. This illustrates the need for a simultaneous confirmation method since multiple ephedrine compounds are often present in a positive urine sample.

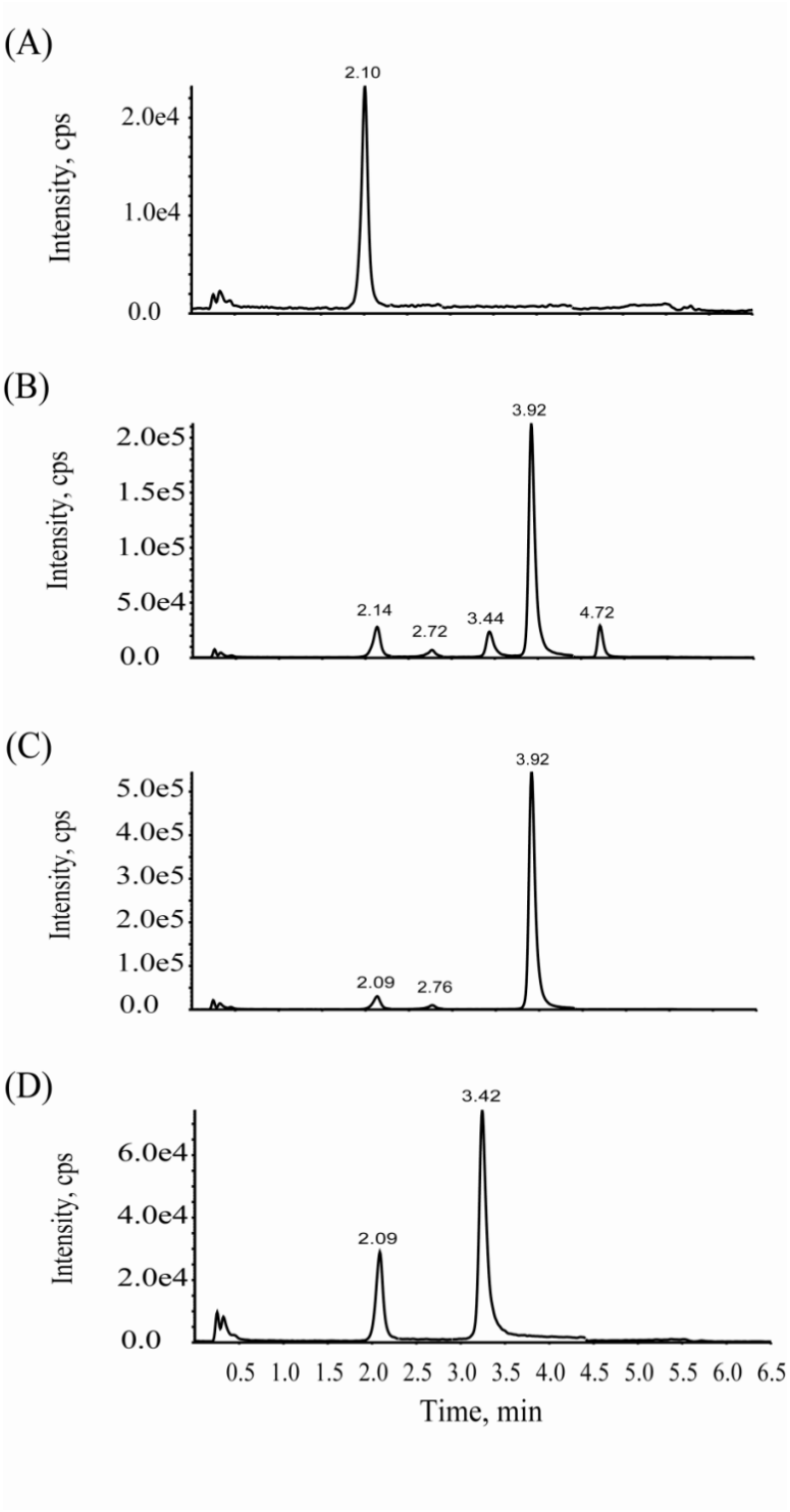


Figure 3.11. Extracted ion chromatograms (XIC) for the analysis a real sample.
A blank (A), a quality control sample (B) and actual samples previously determined positive for cathine and pseudoephedrine (C) and ephedrine (D) was analysed. Separation performed on an XBridge C_{18} 2.5 µm, 50 x 2.1 mm column operated under gradient conditions at 0.5 mL/min and 45 °C.

3.4 Conclusions

The data presented here emphasises the difficulties in selecting eluents optimal for both chromatographic performance and MS ionisation. In this case, the incompatibility was overcome through the use of APCI, which provided a more reliable and reproducible mode of ionisation compared with ESI. The benefits of mobile phase pH optimisation for maximum MS performance, in addition to chromatographic performance, have been illustrated. Results from the flow injection studies with various mobile phase additives using positive ESI confirm the significant effect of pH on signal intensity, although no considerable effect on signal stability was noted. For all of the basic analytes studied, the addition of a base or high pH buffer provides significant benefits for LC-MS analysis. Without the use of chromatography, these results illustrate that the increase in MS response with a basic eluent is not a result of later elution in a higher organic eluent. However, in addition to the increase in ESI response seen for all of the basic analytes studied, the inherent enhanced retention of polar bases under high pH conditions does enable improved separation from early eluting matrix interferences to reduce the risk of ion suppression.

A new, simple LC-APCI-MS/MS method has been developed and validated for the identification and quantification of ephedrines in urine for doping control analysis. The use of a high pH mobile phase has allowed for improved chromatographic separation of the basic compounds without undesirable additives or the need for column regeneration required when using highly aqueous mobile phases. A direct dilution and injection approach circumvents the time- and labour-intensive sample preparation associated with current GC-MS methodologies and provides the

sensitivity and selectivity required. Moreover, the method requires a small sample volume and permits the accurate quantification of cathine, ephedrine, pseudoephedrine and methylephedrine in a single injection, the results of which compare favourably with the current GC-MS method.

CHAPTER 4

COMPARISON OF REVERSED-PHASE AND HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHIC FOR THE QUANTIFICATION OF EPHEDRINES USING HIGH RESOLUTION ACCURATE MASS SPECTROMETRY

4.1 Introduction

While reversed-phase liquid chromatography (RPLC) has become the predominant method of choice for bioanalytical separations, the analysis of hydrophilic basic compounds continues to challenge this mode of chromatography. This is a particular problem for quantitative assays where the complex matrices involved are composed of many polar constituents, such as metabolic wastes, biological salts and polar organic compounds. Early elution of analytes, often with interfering endogenous species in biological matrices in the void volume, and asymmetrical peak shape hamper precise and accurate quantification. As previously discussed, under traditional RPLC conditions highly aqueous mobile phases [24, 96, 97] or ion-pairing additives [98, 138, 139] are required for sufficient retention, resolution and adequate peak shape. However, such approaches are associated with stationary phase de-wetting and subsequent phase collapse under highly aqueous conditions and poor desolvation and ion suppression when coupled to mass spectrometric (MS) detection [99].

With the introduction of chemically stable hybrid stationary phase materials, manipulation of mobile phase pH in RPLC represents a powerful tool to suppress analyte ionisation and chromatograph ionisable analytes in their largely neutral forms [48, 100, 101]. The use of a high pH mobile phase with RPLC has previously been investigated to separate the ephedrines (see Table 4.1 for analyte properties), resulting in good chromatographic separation with acceptable retention and peak shape for identification and quantification in urine for doping control analysis [140]. Highlighted in this study, however, it was found necessary to utilise APCI rather than ESI to achieve acceptable quantitative linearity.

Table 4.1. Properties of the compounds considered in this study.

Compound	pK_a*	log*P* **	log*D* (pH 5)***	log*D* (pH 10)***
Phenylpropanolamine (PPA)	9.4	0.81	- 3.59	0.71
Cathine	9.4	0.81	- 3.59	0.71
Ephedrine	9.6	1.05	- 3.55	0.90
Pseudoephedrine	9.8	1.05	- 3.75	0.84
Methylephedrine	9.3	1.74	- 2.56	1.66

* pK_a values obtained from Clarke's Analysis of Drugs and Poisons

** Log*P* values obtained from ChemSpider

*** Log*D* values calculated from pK_a and log*P* values using the equation: $LogD = \log P + \log\left[\frac{1}{(1+10^{pK-pH})}\right]$

Since the introduction of hydrophilic interaction liquid chromatography (HILIC) by Alpert in 1990 [58], this mode of chromatography has received increasing attention as an alternative to RPLC for the retention and separation of polar or ionisable compounds [48, 141-145]. This complementary technique is defined by a hydrophilic stationary phase used with a highly organic mobile phase, providing orthogonal separation mechanisms to RPLC and therefore different selectivity. In addition to the good retention of very polar analytes, the large percentage of organic modifier in the HILIC mobile phase offers enhanced ion spray in ESI-MS and hence increased sensitivity, as well as lower back-pressures for use of faster flow rates or longer columns [146, 147]. The inherent low viscosity mobile phases in HILIC also have a positive influence on the kinetic performance of separations [148]. A previous comparison between a HILIC and a RPLC approach for the separation of the ephedrines in terms of kinetic performance demonstrated that HILIC outperforms a

high pH RPLC system, providing van Deemter curves with lower *C*-term coefficients due to improved diffusion with the low viscosity mobile phase [141, 148]. As referred to previously, this permits the use of higher linear velocities without sacrificing efficiency, measured by the height equivalent to a theoretical plate (*HETP*). The lowest H_{min} values obtained for ephedrine with pH 10 RPLC and HILIC were 7.4 µm and 4.9 µm respectively, highlighting the benefits of HILIC over the RPLC system in this case. Nevertheless, the technique holds certain disadvantages compared with RPLC, in particular regarding the complex and poorly understood retention mechanisms, which complicate the prediction of retention values in HILIC [59, 149, 150]. HILIC has also been associated with limited flexibility and applicability compared with RPLC, long re-equilibration times and problems with sample solubility. However, many studies report the success of HILIC as a powerful alternative where RPLC fails to provide appropriate retention or peak shape [151], and is particularly amenable to applications coupled to MS detection.

Due to its unrivalled selectivity and sensitivity, tandem triple-quadrupole mass spectrometry coupled to HPLC in selected reaction monitoring mode has traditionally been the primary tool for screening and quantification in the bioanalytical arena. However, with improved modern technologies, high resolution accurate mass spectrometry (HRAMS) has recently emerged as an attractive alternative for simultaneous qualitative and quantitative analysis [22, 152, 153]. Hybrid quadrupole time-of-flight (QTOF) instruments now offer high resolution and mass accuracy, fast scan speeds and MS/MS capabilities, together with improved linear dynamic range and sensitivity. In doping control analysis, several publications report the shift to HRAMS for high-throughput screening [25, 28, 93, 95] and

quantitative analysis [24, 154-156] of small molecules in complex matrices. In addition, high resolution full scan data does not require any prior knowledge of the target analytes or MS/MS parameter optimisation and allows retrospective generation of extracted ion chromatograms.

This study will directly compare high pH RPLC and HILIC conditions for accurate and robust quantitative LC-MS analysis of ephedrines for the purpose of doping control. The relative merits and limitations of each approach are evaluated, comparing retention, peak shape, resolution, linearity, accuracy, and precision and matrix interferences. In addition, there is a focus on the potential of a medium-resolution (10,000 full width at half maximum (FWHM)) accurate mass instrument for the quantitative analysis of small molecules in a biological matrix.

4.2 Experimental

4.2.1 Materials

Acetonitrile (LC-MS grade) and methanol (HPLC grade), propan-2-ol (HPLC grade), ammonium bicarbonate, ammonium hydroxide solution (35 % w/w), formic acid (99-100 %) and trifluoroacetic acid (TFA, ≥ 98.5 %) were obtained from Fisher Scientific (Loughborough, UK). Ammonium acetate was purchased from Sigma (Poole, UK). Norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine and methylephedrine were purchased as hydrochloride salts from Sigma (Poole, UK). Norephedrine-d_3 (used as internal standard) was purchased as a free base (1 mg/mL in methanol) from LGC Standards (Teddington, UK). Water was purified by an ultra-pure water system (Millipore, UK).

4.2.2 Solutions

4.2.2.1 Reversed-phase mobile phase

Ammonium bicarbonate buffer (10 mM) was prepared in purified water and adjusted to pH 10 with ammonium hydroxide solution (35 % w/w).

4.2.2.2 HILIC mobile phase

Mobile phases were pre-mixed using a 200 mM stock buffer of ammonium acetate prepared in purified water and adjusted to pH 5. Mobile phase A consisted of 95:5 v/v CH_3CN:200 mM buffer prepared by taking 50 mL of the 200 mM stock buffer using a glass pipette and adding to 950 mL acetonitrile. Mobile phase B consisted of 40:55:5 v/v/v CH_3CN:H_2O:200 mM buffer prepared by taking 50 mL of

the 200 mM stock buffer and adding to 550 mL purified water and 400 mL acetonitrile. The pre-mixed mobile phase was then sonicated and allowed to reach room temperature to ensure complete dissolution of the buffer salt in the organic phase.

4.2.3 Sample preparation and pre-treatment

Stock solutions were prepared at a concentration of 100 µg/mL for norpseudoephedrine, 1 mg/mL ephedrine and methylephedrine and 10 mg/mL for pseudoephedrine in methanol and stored at -20 °C. A stock solution of norephedrine-d_3, used as an internal standard (IS), was prepared at 10 µg/mL in methanol. Standard working solutions were prepared by diluting stock solutions with the appropriate mobile phase for either reversed-phase or HILIC operation.

A two-step dilution of urine samples was performed before injection: firstly a 45-fold dilution with mobile phase was performed, after which samples were vortexed and centrifuged. Aliquots (200 µL) were then mixed with an equal volume of IS solution (norephedrine-d_3, 500 ng/mL diluted in mobile phase). Vials were vortexed before being placed in the autosampler.

4.2.4 LC conditions

4.2.4.1 Reversed-phase conditions

All separations were carried out on an Acquity UPLC® system (Waters Corp., Milford, USA), with reversed-phase separations performed on an Acquity BEH C_{18}

1.7 µm, 2.1 x 50 mm column equipped with a 0.2 µm in-line filter. The mobile phase consisted of 10 mM ammonium bicarbonate adjusted to pH 10 (A) and methanol (B). Gradient chromatography was performed starting at 10 % B, increasing to 25 % B after 2.70 min, 55 % B at 5.00 min, 95 % B at 5.50 min and returning to 10 % B for a 1 min re-equilibration. The flow rate was 0.6 mL/min and the column temperature was set at 45 °C. The weak and strong needle wash lines of the Acquity UPLC® system were placed in 90:10 v/v H_2O:CH_3OH (v/v) and 60:30:10 v/v/v C_3H_8O:CH_3CN:H_2O with 0.1% TFA, respectively. The injection volume was 2 µL performed in full loop mode. This UPLC® method was modified from a previously reported HPLC separation [140].

4.2.4.2 HILIC conditions

The HILIC separation was performed on an Acquity BEH HILIC 1.7 µm, 2.1 x 100 mm column (Waters, Milford USA) using a 0.2 µm in-line filter and isocratic conditions. The mobile phase comprised pre-mixed 95:5 v/v CH_3CN:200 mM ammonium acetate pH 5 (A) and 50:45:5 v/v/v CH_3CN:H_2O:200 mM ammonium acetate. A flow rate of 0.5 mL/min was used and the column temperature was set at 50 °C. The weak and strong needle wash lines of the Acquity UPLC® system were placed in 95:5 v/v CH_3CN:H_2O and 50:50 v/v CH_3CN:H_2O respectively, so as not to cause any interference with the sample plug. The injection volume was 2 µL performed in full loop mode.

4.2.5 Mass spectrometry

Mass spectrometric detection was performed using a Waters Xevo QTOF instrument operated in positive mode with ESI. The MS data acquisition was performed in MS^E mode, collecting two channels of data throughout the run, one with low collision energy (4 V) and one high (ramp from 10-20 V) in order to obtain both the precursor and product ions. Source conditions were optimised for each mobile phase composition and, for the reversed-phase separation, the capillary voltage was set at 0.5 kV, sample cone 15 V and extraction cone 2.0 kV. In HILIC the capillary voltage was set at 0.7 kV, sampling cone 15 V and extraction cone 4.0 kV. For both LC setups the source temperature was 120 °C, desolvation temperature was 500 °C, cone gas flow was set at 10 L/h and the desolvation gas was set at 800 L/h. The micro-channel plate detector was operated at 2275 V. Data was collected in centroid mode over an *m/z* range of 50-600 Da with a scan time of 0.1 sec. Leucine enkephalin (5 ng/mL) was used as the accurate mass reference material, infused through a LockSpray probe at 5 µL/min and data was acquired every 30 seconds with a 0.5 sec scan time (3 scans were averaged). A mass window of 0.005 Da was used to extract mass traces. MassLynx^{TM} V4.1 software (Waters Corp., Milford, USA) was used for data acquisition and analysis.

4.2.6 Calibration

A six-point calibration curve including 50-200 % of the WADA threshold concentrations was constructed from a stock calibration solution containing cathine 10 µg/mL, ephedrine 20 µg/mL, pseudoephedrine 300 µg/mL and methylephedrine 20 µg/mL spiked into blank urine. This was diluted to form the subsequent

calibration points at 1.25, 2.5, 3.75, 5.0, 7.5, 10.0 µg/mL for cathine, 2.5, 5.0, 7.5, 10.0, 15.0 and 20.0 µg/mL for ephedrine and methylephedrine and 37.5, 75.0, 112.5, 225 and 300 µg/mL for pseudoephedrine. Quality control (QC) samples were prepared at concentrations equal to the WADA threshold levels (cathine 5 µg/mL, ephedrine 10 µg/mL, pseudoephedrine 150 µg/mL, methylephedrine 10 µg/mL).

4.2.7 Validation

The validation was performed on three different days, with mobile phase, calibrants and QCs prepared on each individual day. The method was validated for linearity over the dynamic range, selectivity, accuracy, precision, carry-over and ion suppression due to matrix interferences. Ten blank urine samples obtained from different volunteers were analysed as described above to ensure selectivity of the method. Linearity was determined with the six-point calibration and accuracy and between-assay precision were determined by analysing three replicates of a urine sample spiked with each analyte at the threshold concentration on each day.

4.3 Results

The use of a high pH mobile phase was previously investigated for the separation of ephedrines by RPLC to suppress ionisation of the basic analytes and inhibit undesirable secondary interactions with residual silanols on the surface of the stationary phase [140]. Although this approach enabled improved separation and peak shape compared with traditional low pH RPLC methods, some important factors in coupling solvent systems optimal for LC separation with mass spectrometric (MS) detection were highlighted. When interfaced with MS detection it suffered from problems with poor ESI spray and non-linear, irreproducible data. APCI was therefore employed to overcome this and the resulting LC-APCI-MS method was validated. However, here HILIC is evaluated as an alternative approach for the separation of the polar and ionisable diastereoisomeric pairs, well known for its advantages when coupled to MS detection. The parameters affecting HILIC separation including organic content, buffer concentration, pH and temperature have been previously optimised for the separation of ephedrines for an evaluation of kinetic performance and these conditions are used here as the comparative method [148]. Although speed of analysis is a critical factor in method development, the main objective here was to develop the most accurate, precise and robust quantification assay for ephedrines in doping control analysis. Some considerations for HILIC method development are discussed and a comparison is made between the high pH RPLC and the novel HILIC approach for the quantification of ephedrines.

4.3.1 Comparison of chromatographic parameters

The previously reported RPLC method has been modified to enable transfer from HPLC (Waters XBridge C_{18} 2.5 μm, 50 x 2.1 mm i.d.) to UPLC® (Waters BEH C_{18} 1.7 μm, 50 x 2.1 mm i.d.) technology. The final chromatographic conditions of the methods compared here are detailed in Table 4.2 and the resulting chromatograms in Figure 4.1 with the corresponding chromatographic parameters in Table 4.3. Although both approaches show adequate retention of all analytes, the pH 10 RPLC provides stronger retention (*k* values ranging from 6.0-15.5) despite the use of gradient chromatography compared with the HILIC isocratic method (*k* values ranging from 4.3-6.9). With RPLC, operating at high pH suppresses analyte ionisation which enables hydrophobic interactions to dominate the mechanisms of retention. In contrast, under HILIC conditions at pH 5, where the basic analytes are fully ionised, this protonation aids interaction with the aqueous layer on the surface of the silica as well as ionic interactions with exposed silanols on the polar stationary phase. It is interesting to note that the elution order only changes with respect to methylephedrine; it is not reversed for the other ephedrines between RPLC and HILIC, indicating that the two approaches are not orthogonal. The pH 10 RPLC offers greater resolution between each of the analytes, with a minimum Rs value of 2.4, compared with the HILIC method with a minimum Rs value of 1.8. Nevertheless, HILIC offers the major advantage of improved peak shape, essential for accurate integration and therefore quantification. Additionally, with the low viscosity mobile phase used under HILIC conditions, the possibility of using a longer column for enhanced resolution is not restricted by back-pressure limitations.

Table 4.2. Chromatographic conditions of the final RPLC pH 10 and HILIC methods used in this comparative study.

	RPLC pH 10			HILIC		
Column	Waters BEH C_{18} 1.7 µm, 50 x 2.1 mm i.d.			Waters BEH HILIC 1.7 µm, 100 x 2.1 mm i.d.		
Mobile phase A	10 mM ammonium bicarbonate pH 10			40:55:5 CH_3CN:H_2O:200 mM ammonium acetate pH 5		
Mobile phase B	CH_3OH			95:5 CH_3CN:200 mM ammonium acetate pH 5		
Flow rate	0.6 mL/min			0.5 mL/min		
Column temp.	45 ºC			50 ºC		
Injection vol.	2 µL			2 µL		
Gradient	**Time (min)**	**A (%)**	**B (%)**	**Time (min)**	**A (%)**	**B (%)**
	0.0	90	10	0.0	0	100
	2.70	75	25	5.0	0	100
	5.00	45	55	5.5	100	0
	5.50	5	95	6.0	0	100
	6.5	90	10	10.0	0	100

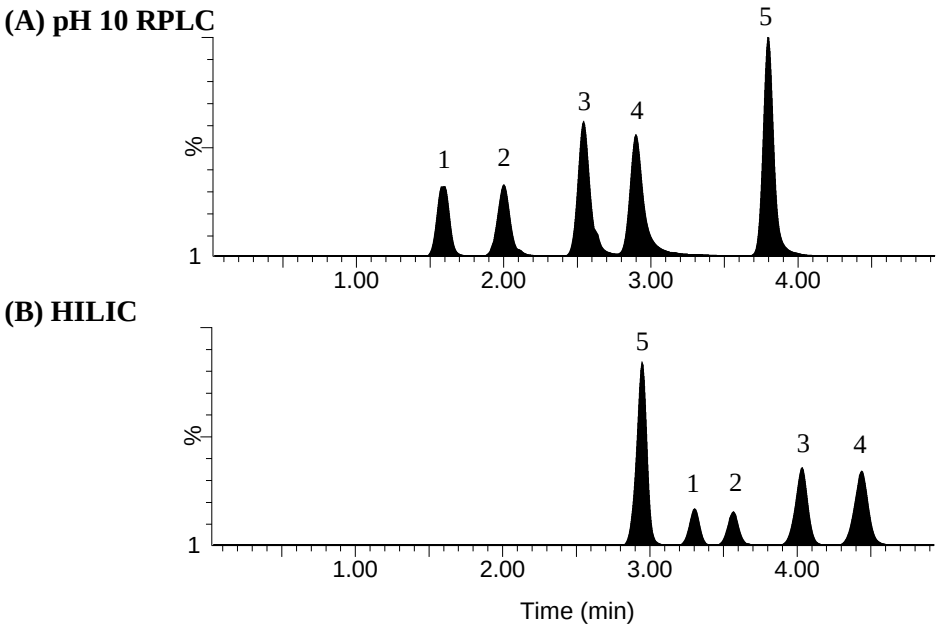


Figure 4.1. Comparison of pH 10 RPLC (A) and HILIC (B).
Chromatograms illustrate separation of PPA (1), cathine (2), ephedrine (3), pseudoephedrine (4) and methylephedrine (5) generated using standards prepared at 500 ng/mL in the appropriate mobile phase. RPLC performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column operated under gradient conditions at 0.6 mL/min and 45 °C. HILIC separation performed on an Acquity BEH HILIC 1.7 µm, 100 x 2.1 mm column operated under isocratic conditions at 0.5 mL/min and 50 °C.

Table 4.3. Chromatographic parameters of RPLC and HILIC approaches.
Analytes were injected are at the same concentrations (500 ng/mL) diluted in the appropriate mobile phase.

Analyte	RPLC						
	w_b (min)	$w_{5\%}$ (min)	t_R (min)	t_0 (min)	k	Rs	α
d_3-norephedrine	0.13	0.12	1.60	0.23	6.0	-	-
Cathine	0.15	0.14	2.00	0.23	7.7	2.9	1.3
Ephedrine	0.15	0.14	2.55	0.23	10.1	3.7	1.3
Pseudoephedrine	0.15	0.14	2.91	0.23	11.7	2.4	1.2
Methylephedrine	0.12	0.11	3.80	0.23	15.5	6.6	1.3

Analyte	HILIC						
	w_b (min)	$w_{5\%}$ (min)	t_R (min)	t_0 (min)	k	Rs	α
d_3-norephedrine	0.15	0.14	3.35	0.57	4.9	2.3	1.1
Cathine	0.16	0.15	3.63	0.57	5.4	1.8	1.1
Ephedrine	0.19	0.18	4.07	0.57	6.1	2.5	1.1
Pseudoephedrine	0.20	0.19	4.48	0.57	6.9	2.1	1.1
Methylephedrine	0.15	0.14	3.00	0.57	4.3	-	-

4.3.2 HILIC robustness and reproducibility

Despite the advantages of the HILIC method, replicating the separation was a challenge due to variable retention times, co-eluting and even distorted peaks. Modification of the surface silica from a build-up of matrix contaminants from the injection of diluted urine was initially considered to be the cause, resulting in peak broadening, peak tailing, retention time drift and split peaks. The importance of adequate column conditioning with up to 10 injections of sample matrix before a run has been reported elsewhere to avoid changes in retention throughout a run [157]. The unbonded hybrid silica used here was washed before and after use with 50:50 CH_3CN:H_2O and equilibrated for up to an hour with the mobile phase to ensure good establishment of the water layer on the surface. Several blank urine samples were injected to condition the column and ensure the matrix was not contributing to

changes in retention. Following the isocratic elution, a wash gradient up to 40:60 CH_3CN:aqueous buffer was employed to remove any polar matrix components remaining on the silica surface. These measures ensured that there was no surface modification or matrix interference and that direct injection was suitable, omitting the need for extensive time-consuming sample preparation procedures.

4.3.3 HILIC mobile phase preparation

The adopted mobile phase from the previous study utilises a buffered acetonitrile eluent. This was used in order to control the extent of electrostatic interaction between ionised analytes and silanols on the silica surface. Moreover, it is important to maintain the eluent pH and a constant charge state of ionisable analytes to ensure retention reproducibility. The previous method development study detailed the high sensitivity of retention to the slightest change in pH and/or percentage of organic modifier. Meticulous preparation is therefore necessary to ensure reproducible retention. The 200 mM aqueous ammonium acetate stock buffer, adjusted to pH 5, was stored at 4 ˚C and left to reach room temperature and the pH then measured again. To prepare 1 L of mobile phase, 50 mL of 200 mM stock buffer was measured using a glass pipette and 950 mL of acetonitrile was added. This was sonicated for 10 minutes to ensure complete dissolution of the ammonium acetate in the acetonitrile. The exact measurement of each of these components was found to be critical in reproducing the same retention times and separation. Once the separation had been replicated, this same procedure was repeated several times on different days, using different preparations of stock buffer, in order to evaluate the reproducibility of this approach.

4.3.4 Retention time reproducibility

Poor reproducibility can be a major disadvantage associated with HILIC. This was apparent in the present study where several factors were investigated as potential causes of variation. Mobile phase preparation and storage, column conditioning, re-equilibration times and injection solvent were all considered critical. Retention time reproducibility deteriorated over time when mobile phase was not prepared daily and left stored on the instrument. This resulted in retention drifts as large as 0.2 min (Figure 4.2), whereas for mobile phase stored in tightly sealed bottles, retention was stable for more than one week.

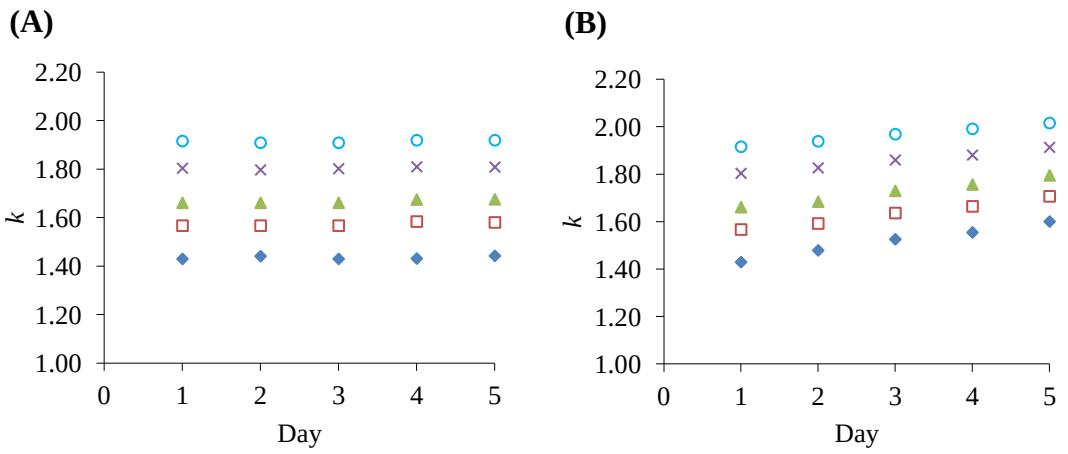


Figure 4.2. Change in retention factors over 5 days with different storage conditions. Solvent reservoirs were stored tightly capped (A) or with a duran stopper (B) and retention changes evaluated for methylephedrine (♦), PPA-d3 (□), cathine (▲), ephedrine (x), and pseudoephedrine (○).

The increase in analyte retention, seen in Figure 4.2, is thought to be related to the pH changes over time when the mobile phase is left exposed, with evaporation of the organic phase expected to result in stronger elution in HILIC. In HILIC, a retention increase is observed as the amount of organic is increased, hence this increase in retention must be the result of a change in pH and not evaporation of acetonitrile

alone. Therefore, the possibility that mobile phase pH is affected by the evaporation of acetonitrile with the pre-mixed volatile ammonium acetate buffer was investigated. The apparent pH of the mobile phase was measured on each day for 12 consecutive days by storing mobile phase both tightly sealed and left open. The results indicate an apparent pH change occurring when the HILIC mobile phase is exposed, an effect which is not seen in the pure aqueous buffer containing no organic modifier (Figure 4.3). This supports the proposed theory that evaporation of acetonitrile causes a decrease in the apparent pH, bringing it closer to that of the pure aqueous buffer. An additional consideration is that the decrease in pH is due to carbon dioxide absorption from the solvent reservoir, leading to the formation of carbonic acid and decrease in pH. However, since the change in pH is only noted in buffer containing acetonitrile, this evidence suggests that evaporation of the organic portion is accountable for the decrease in pH.

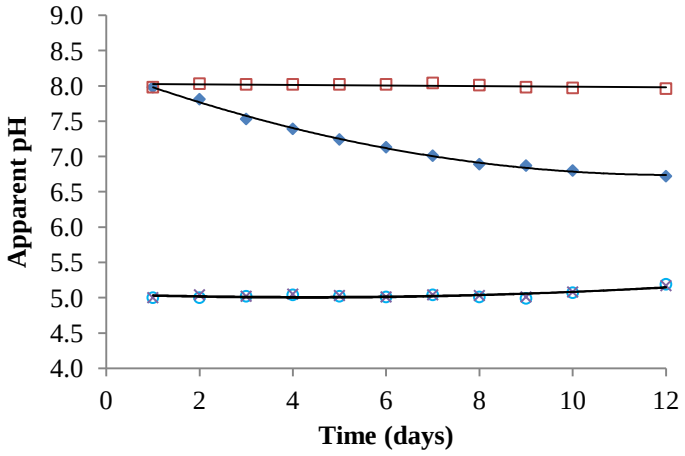


Figure 4.3. Change in apparent pH of HILIC mobile phase.
Apparent pH was measured daily for mobile phase (95:5 AcN:10 mM ammonium acetate pH 5) was stored tightly capped (□) and with a Duran stopper (♦) and aqueous 10 mM ammonium acetate pH 5 stored tightly capped (x) and with a Duran stopper (○).

However, if mobile phase is freshly prepared and stored appropriately, retention times have been shown to be stable and reproducible. Variation was calculated on three separate days using three different preparations of buffer and mobile phase. The results are expressed in Table 4.4 and show that inter-day retention times do not vary by greater than 0.17 %.

Table 4.4. Retention times of analytes spiked in urine on three different days using three different preparations of mobile phase, n = 6.

		Day 1	Day 2	Day 3	Inter-day
Methylephedrine	**Ave (min)**	2.98	3.01	2.98	2.99
	STDEV	0.01	0.01	0.00	0.01
	% RSD	0.21	0.20	0.11	0.17
Norephedrine-d_3	**Ave (min)**	3.37	3.39	3.37	3.38
	STDEV	0.01	0.00	0.00	0.00
	% RSD	0.23	0.01	0.08	0.11
Cathine	**Ave (min)**	3.56	3.58	3.57	3.57
	STDEV	0.00	0.00	0.00	0.00
	% RSD	0.00	0.01	0.12	0.04
Ephedrine	**Ave (min)**	4.02	4.04	3.99	4.02
	STDEV	0.01	0.00	0.00	0.00
	% RSD	0.20	0.00	0.01	0.07
Pseudoephedrine	**Ave (min)**	4.43	4.45	4.39	4.42
	STDEV	0.00	0.01	0.00	0.00
	% RSD	0.01	0.17	0.00	0.06

It has been documented that HILIC requires longer re-equilibration times than RPLC, typically 10-20 column volumes, and was therefore optimised following the wash gradient to permit stable retention. At a flow rate of 0.5 mL/min, four minutes re-equilibration was suitable for chromatographic stability and repeatable retention. Retention was reproducible with as little as two minutes re-equilibration, but the elution of matrix interferences showed poor stability and inconsistent retention

resulted in co-elution with the analytes and inaccurate quantification. This can be seen in

Figure 4.4, which indicates the change in retention of matrix components over consecutive injections of the same spiked sample (highlighted with *). This is thought to be caused by the steep wash gradient at the end of the run as this phenomenon is not seen when operation is performed solely in the isocratic mode. The problem was rectified by extending the re-equilibration time, the results of which can be seen in Figure 4.5 where the interference (highlighted with *) is eliminated when a four minute re-equilibration is employed. This is considerably longer than the 1.5 minute re-equilibration for the pH 10 RPLC method, resulting in final run-time of 10 minutes for the HILIC method compared with 6.5 minutes for the pH 10 RPLC method. This is a clear disadvantage of the HILIC approach as the throughput is significantly poorer than for the RPLC approach, and should be taken into consideration when turnaround time is of concern.

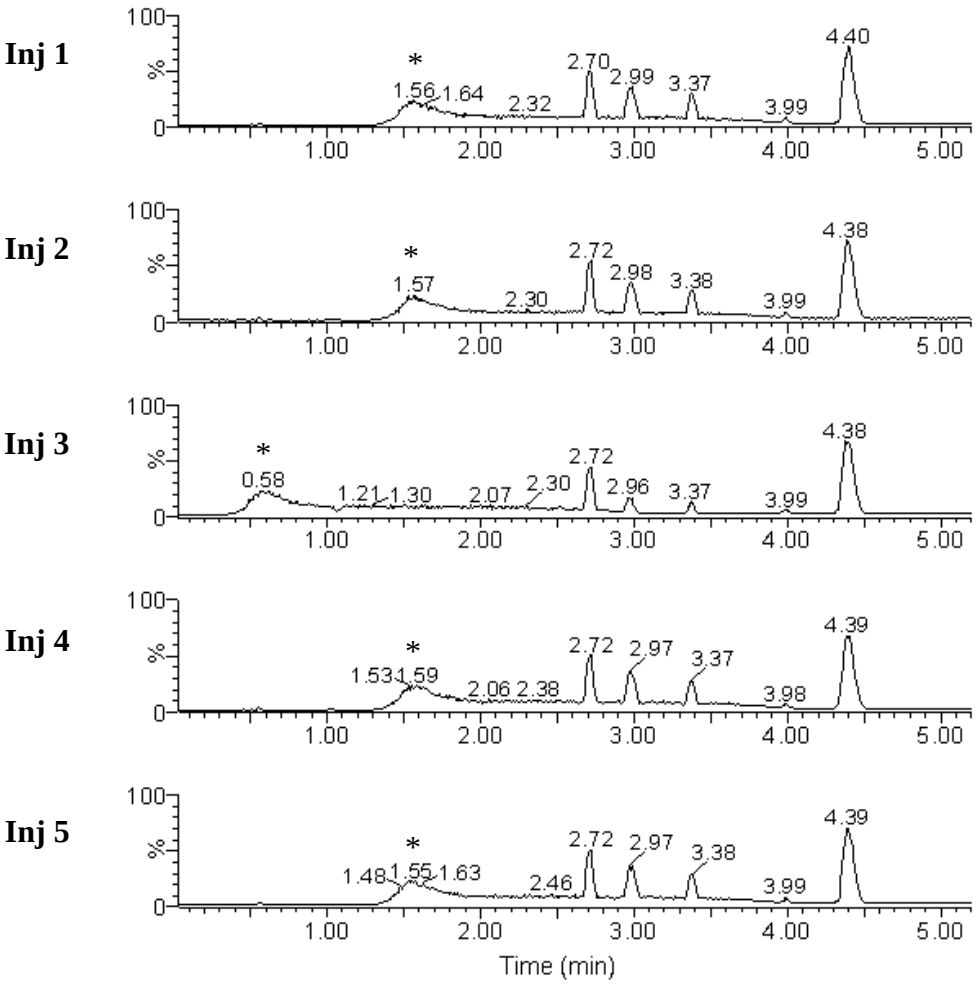


Figure 4.4. Repeat injections of a spiked urine sample indicating change in the retention of matrix interferants between consecutive injections.
Experiments performed on an Acquity BEH HILIC 1.7 µm, 100 x 2.1 mm column operated at 0.5 mL/min and 50 °C. Matrix interferants highlighted with *.

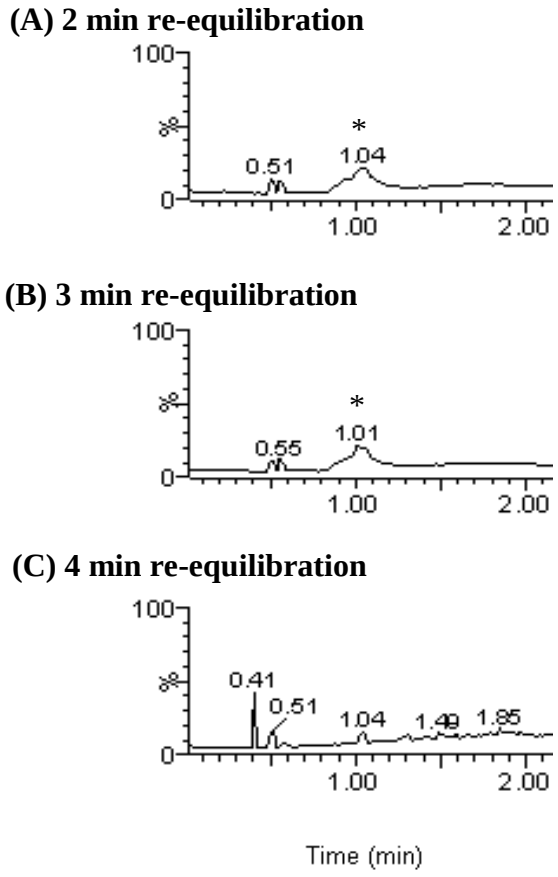


Figure 4.5. Effect of increasing re-equilibration time on matrix interferants seen with diluted blank urine.
Experiments performed on an Acquity BEH HILIC 1.7 µm, 10 x 2.1 mm column operated at 0.5 mL/min and 50 °C. Matrix interferants highlighted with *.

4.3.5 Loading

The sample loading capacity of reversed-phase type B silica columns has been extensively reported, portraying poor loading capacity of basic compounds under acidic conditions [104-106]. This has been attributed to rapid saturation of exposed negatively charged silanol groups on the surface of the stationary phase. However, it has been shown that under high pH conditions where basic analytes are largely unionised the loading capacity is much improved, with higher concentrations tolerated before the characteristic "shark fin" peak shape is noted [107]. Here, the loading capacity of the HILIC BEH material is compared to the BEH C_{18} material under basic conditions. The results are illustrated in Figure 4.6, which depicts the

preservation of Gaussian peak shape under HILIC conditions with increasing sample load compared with pH 10 RPLC conditions. Although high pH mobile phase offers a significant advantage over traditional acidic RPLC conditions in terms of improved retention and peak shape with increasing sample [104, 140], HILIC was better able to maintain retention time and symmetrical peak shape with increases in the amount of sample on column compared with RPLC at pH 10. This is illustrated in Figure 4.6 for pseudoephedrine, where at 500 ng on column, HILIC gave an asymmetry value at 10 % peak height ($As_{10\%}$) of 1.67 compared with 2.39 using pH 10 RPLC. Retaining peak shape with large amounts of solute on column is particularly beneficial for this application where there is great interest in analysing the lower threshold level ephedrines (cathine, ephedrine and methylephedrine) together with pseudoephedrine which is likely to be present at much higher concentrations.

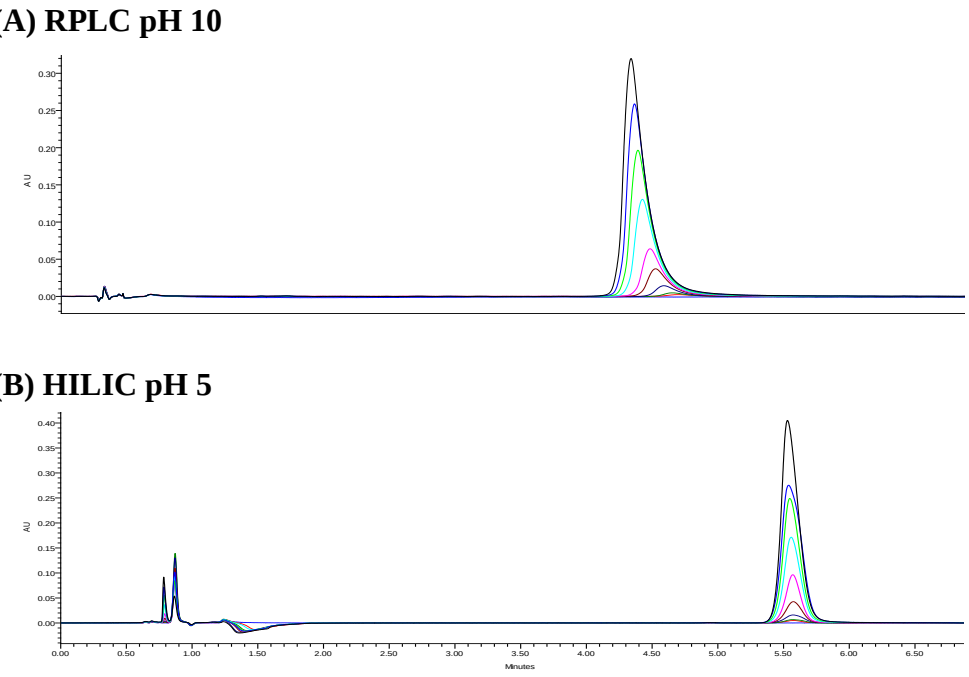


Figure 4.6. Overlay of pseudoephedrine with increasing concentration under RPLC pH 10 (A) and HILIC pH 5 (B) conditions.
Pseudoephedrine injected at 20, 50, 100, 200, 250, 300, 400 and 500 ng on column under high pH RPLC (A) and HILIC conditions (B) at 210 nm. RPLC performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column; HILIC performed on an Acquity BEH HILIC 1.7 µm, 100 x 2.1 mm column.

4.3.6 Quantification using high resolution accurate mass

A transition from the use of tandem mass spectrometry to high resolution accurate mass spectrometry (HRAMS) has recently been adopted in drug discovery and screening laboratories to enable full scan accurate mass data acquisition. The introduction of fast scanning quadrupole time-of-flight (QTOF) instrumentation represents an attractive tool for comprehensive screening and confirmation of analytes coupled to UHPLC. Modern HRAM technologies, equipped with enhanced sensitivity and linear dynamic range, offer the potential for quantitative analysis together with confirmation via accurate mass measurements, analyte elemental composition and the high selectivity required for analysis of complex matrices. With the QTOF mass analyser used here it was possible to acquire two channels of data with different collision energies to obtain both molecular and fragment ion data for confirmation. A low collision energy (CE = 4V) was applied in one channel to obtain molecular ions and in the other a higher collision energy (CE = 10-20V ramp) to generate fragmented ions (Figure 4.7). In addition, the improved sensitivity and dynamic range allowed for simultaneous quantification, with good linearity, accuracy and precision over the calibration range.

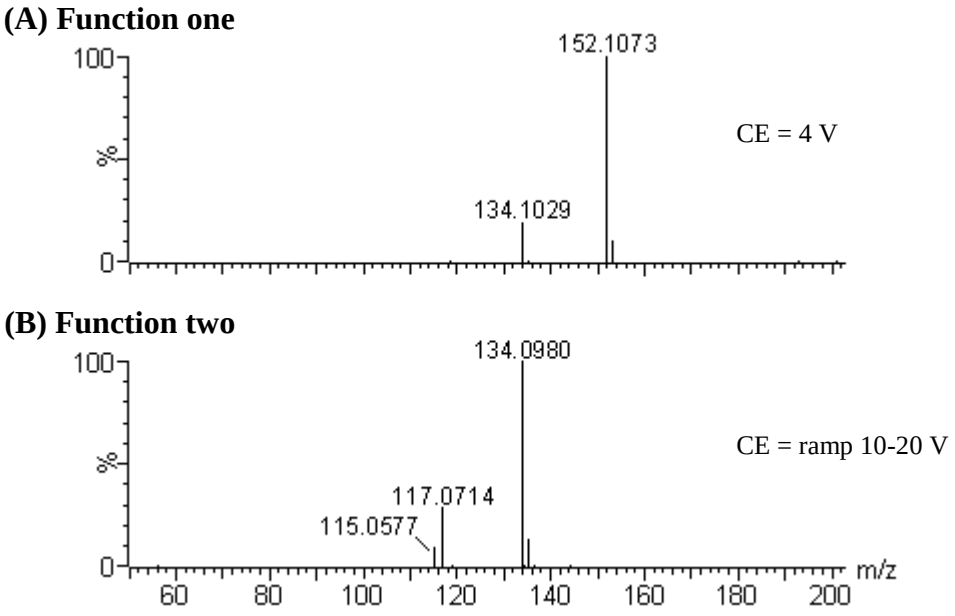


Figure 4.7. Mass spectra of function one (molecular ions) and function two (fragment ions) for cathine.

The ions used for quantification and identification are listed in Table 4.5. The protonated molecules obtained from the low collision energy channel were used in quantification, while the additional fragment ions created in the high collision energy channel were included for confirmation analysis.

A scan time of 0.1 sec/scan was used here, optimised to give sufficient data points for good peak shape definition and mass accuracy. With both the RPLC and HILIC methods, a sufficient number of scans were obtained per peak for quantification (≥ 18 data points per peak). This is especially important for quantification, where 12-15 data points per peak are required for good peak definition accurate peak area measurement. On each day of the validation, accurate mass measurements were performed and the errors between the expected and measured mass values (mDa and ppm) were calculated, given in Table 4.5.

Table 4.5. Protonated molecules with calculated accurate mass values and errors of the analytes used for quantification (n = 6) and their fragment ions used for confirmation.

Compound	Molecular formula	Accurate mass $[M+H]^+$	Average measured mass $[M+H]^+$	Average mass accuracy		Fragment ions		
				(mDa)	(ppm)			
Norephedrine-d_3	$C_9H_{10}D_3NO$	155.1264	155.1262	-0.2	-1.4	137.2168 ($C_9H_9D_3N$)	117.0704 (C_9H_9)	115.0548 (C_8H_7)
Cathine	$C_9H_{13}NO$	152.1075	152.1063	-1.0	-6.5	134.0970 ($C_9H_{12}N$)	117.0704 (C_9H_9)	115.0548 (C_8H_7)
Ephedrine	$C_{10}H_{15}NO$	166.1232	166.1218	-1.2	-7.0	148.1126 ($C_{10}H_{14}N$)	133.0891 ($C_9H_{11}N$)	117.0704 (C_9H_9)
Pseudoephedrine	$C_{10}H_{15}NO$	166.1232	166.1227	-0.5	-3.1	148.1126 ($C_{10}H_{14}N$)	133.0891 ($C_9H_{11}N$)	117.0704 (C_9H_9)
Methylephedrine	$C_{11}H_{17}NO$	180.1388	180.1382	-0.7	-3.6	162.1283 ($C_{11}H_{16}N$)	147.1048 ($C_{10}H_{13}N$)	135.1174 ($C_{10}H_{15}$)

4.3.7 Validation

Once shown to be reproducible, a validation of the quantitative HILIC method was performed to evaluate linearity, accuracy and precision, repeatability, specificity and matrix effects – and the method applied to a real sample. The same process was applied to the high pH RPLC UHPLC method transferred and modified from the previously validated HPLC version, and the results were compared for a reliable and robust quantification method.

4.3.7.1 Linearity

A calibration curve was generated covering 50-200 % of the WADA threshold concentrations for the prohibited ephedrines (cathine 5 µg/mL, ephedrine 10 µg/mL, pseudoephedrine 150 µg/mL, methylephedrine 10 µg/mL). The use of a high pH RPLC method has previously been reported where difficulty in coupling to ESI-MS detection was highlighted. This was noted again here with pseudoephedrine showing non-linearity and poor reproducibility over the calibration range. Reducing the concentration of the pseudoephedrine calibrants from 37.5-300 µg/mL to 3.75-30 µg/mL, bringing them into the same dynamic range as the other analytes, remedied this phenomenon, indicating that at higher concentrations some peak tailing resulting from a degree of column overloading is still apparent, complicating peak integration. The results, shown in Table 4.6, illustrate the differences obtained with RPLC pH 10 and HILIC.

Table 4.6. Average calibration statistics for the RPLC pH 10 (A) and HILIC (B) methods, n = 3.

(A) RPLC pH 10

Compound	Ion (m/z)	Linear Range (µg/mL)	Slope	Intercept	r^2
Cathine	152.1075	1.25-10	0.0187	0.0112	0.9973
Ephedrine	166.1232	2.5-20	0.0512	0.0181	0.9967
Pseudoephedrine	166.1232	3.75-30	0.0402	0.0130	0.9935
Methylephedrine	180.1388	2.5-20	0.0635	0.0253	0.9947

(B) HILIC

Compound	Ion (m/z)	Linear range (µg/mL)	Slope	Intercept	r^2
Cathine	152.1075	1.25-10	0.0290	0.0052	0.9967
Ephedrine	166.1232	2.5-20	0.0585	0.0206	0.9939
Pseudoephedrine	166.1232	37.5-300	0.0304	0.3244	0.9959
Methylephedrine	180.1388	2.5-20	0.1241	0.1555	0.9943

4.3.7.2 Selectivity

Selectivity was determined through the analysis of 10 different blank urine samples with no interference being detected at the expected retention times of the analytes.

4.3.7.3 Accuracy and precision

Accuracy and precision were calculated on three separate days for both techniques using QCs at threshold concentrations for each analyte. The results are detailed in Table 4.7. Accuracy ranged from 0.0-7.5 % and 0.9-7.6 % with RPLC and HILIC, respectively. RSD values for within-assay variation ranged from 1.3-7.0 % (n = 6) with RPLC and 1.8-10.3 % (n = 6) with HILIC and between-assay variation ranged from 0.7-4.9 % (n = 3) with RPLC and 2.8-4.9 % (n = 3) with HILIC.

Table 4.7 Validation data for accuracy and precision, n = 3

Compound		RPLC pH 10				HILIC pH 5			
		Mean conc. (µg/mL)	Accuracy (% bias)	Precision (% RSD)		Mean conc. (µg/mL)	Accuracy (% bias)	Precision (% RSD)	
				Within-assay	Between-assay			Within-assay	Between-assay
Cathine	Day 1	4.81	-3.80	2.36	0.68	4.70	-6.00	1.98	4.93
(5 µg/mL)	Day 2	4.76	-4.80	3.58		5.18	3.53	5.05	
	Day 3	4.81	-3.80	7.00		4.88	-2.40	10.3	
Ephedrine	Day 1	9.34	-6.60	3.12	3.46	9.24	-7.57	1.84	2.84
(10 µg/mL)	Day 2	10.00	0.00	2.44		9.65	-3.50	3.71	
	Day 3	9.81	-1.90	5.57		9.76	-2.40	7.96	
Pseudoephedrine	Day 1	152.4	1.59	2.08	1.64	140.7	-6.21	3.10	4.84
(150 µg/mL)	Day 2	151.4	0.91	1.30		154.4	2.91	5.09	
	Day 3	147.7	-1.53	6.66		151.4	0.94	8.35	
Methylephedrine	Day 1	9.25	-7.50	5.25	4.90	9.54	-4.60	2.10	4.83
(10 µg/mL)	Day 2	10.2	1.90	3.97		10.5	5.00	7.07	
	Day 3	9.6	-3.70	5.93		10.1	0.90	7.61	

4.3.7.4 Carryover

Carryover was determined by injecting urine samples spiked with each analyte at a concentration equivalent to the highest point of the calibration followed by injection of blank mobile phase. The washes were optimised to minimise carryover and the Acquity UPLC® was operated with a 2 µL loop in full loop mode to maximise the strong wash cycle. Following this optimisation, no carryover was observed.

4.3.7.5 Sensitivity

One of the major advantages of using HILIC over RPLC with ESI is the gain in sensitivity owing to improved desolvation from the high organic content of the mobile phase. It was therefore anticipated that HILIC would enable lower limits of detection compared with the RPLC approach. For this application the limits of detection were determined by the lowest point of the calibration curve, so sensitivity was measured by the signal-to-noise ratio. Cathine at the lowest point of the calibration (11.1 ng/mL following dilution), had S/N values for the RPLC pH 10 and HILIC methods of 9.72 and 59.6, respectively, illustrating a 6-fold increase in sensitivity with the HILIC approach (Figure 4.8). Although sensitivity is not a problem for these high threshold level compounds, a gain in sensitivity permits a larger dilution of sample to reduce the possibility of sample overload and detector saturation, and also minimises matrix interference. The gain in sensitivity, however, becomes interesting when analysing low level analytes, or where longer windows of detection are required.

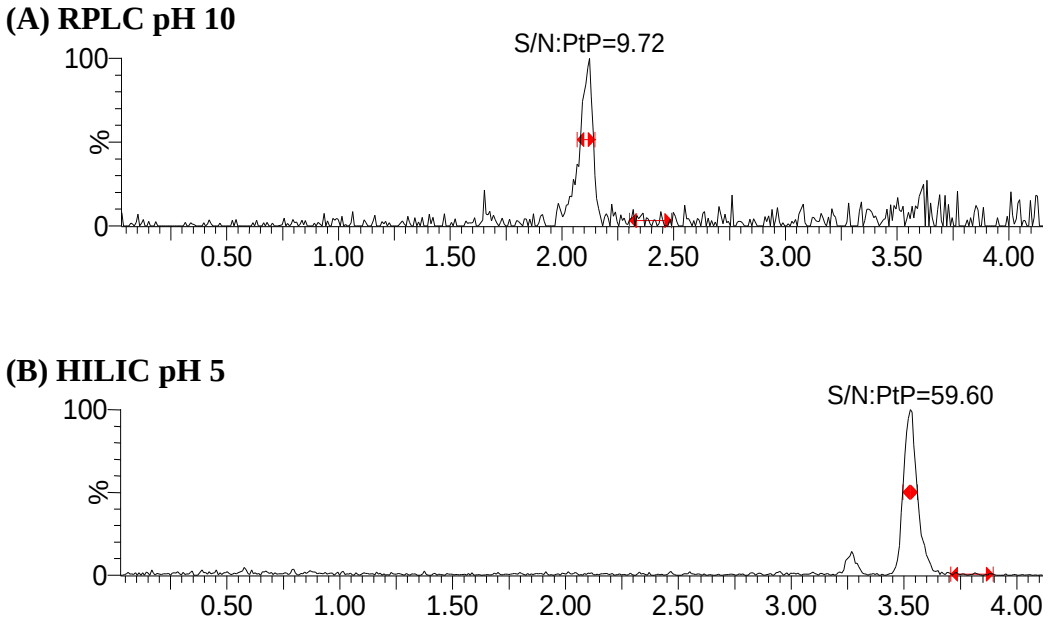


Figure 4.8. Extracted ion chromatograms (XIC) illustrating the difference in sensitivity with RPLC pH 10 (A) and HILIC (B).
Chromatograms illustrate difference in sign-to-noise ratio (S/N) for cathine at the lowest point of the calibration (mass 152.1075 Da with a 0.02 Da mass window). RPLC performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column operated under gradient conditions at 0.6 mL/min and 45 °C. HILIC separation performed on an Acquity BEH HILIC 1.7 µm, 100 x 2.1 mm column operated under isocratic conditions at 0.5 mL/min and 50 °C.

4.3.7.6 Matrix effects

Both approaches were evaluated for matrix interference using 10 different blank urine samples spiked at WADA threshold concentration levels with each analyte considered. Matrix effect was calculated by comparing the response of each analyte spiked in urine samples to standards prepared in mobile phase. Ion suppression from possible matrix interference was shown to be negligible for both methods, with maximum matrix effect values of 106.2 % and 87.0 % for RPLC pH 10 and HILIC, respectively (Figure 4.9). Response factor plots of peak area/concentration versus concentration are also given in Figure 4.10 to evaluate low level matrix effects. The lower the r^2 values of these plots the lower the matrix interference. It can be seen

from these plots, therefore, that in this case RPLC is affected slightly more by matrix interferences than is HILIC.

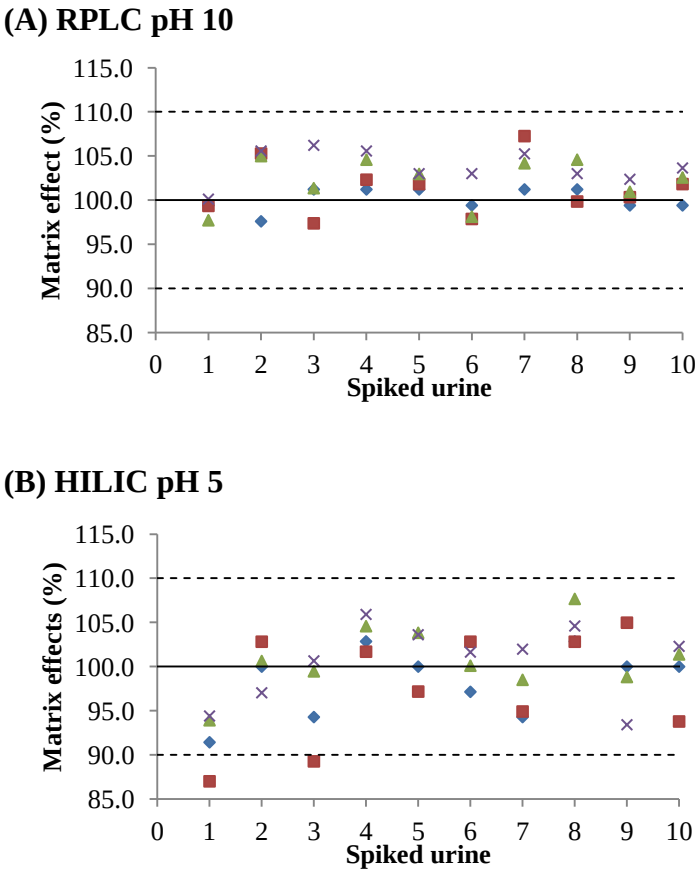


Figure 4.9. Graphical representation of matrix effects.
10 different blank urine samples were evaluated using RPLC pH 10 (A) and HILIC pH 5 (B). Cathine (♦), ephedrine (□), pseudoephedrine (▲) and methylephedrine (x).

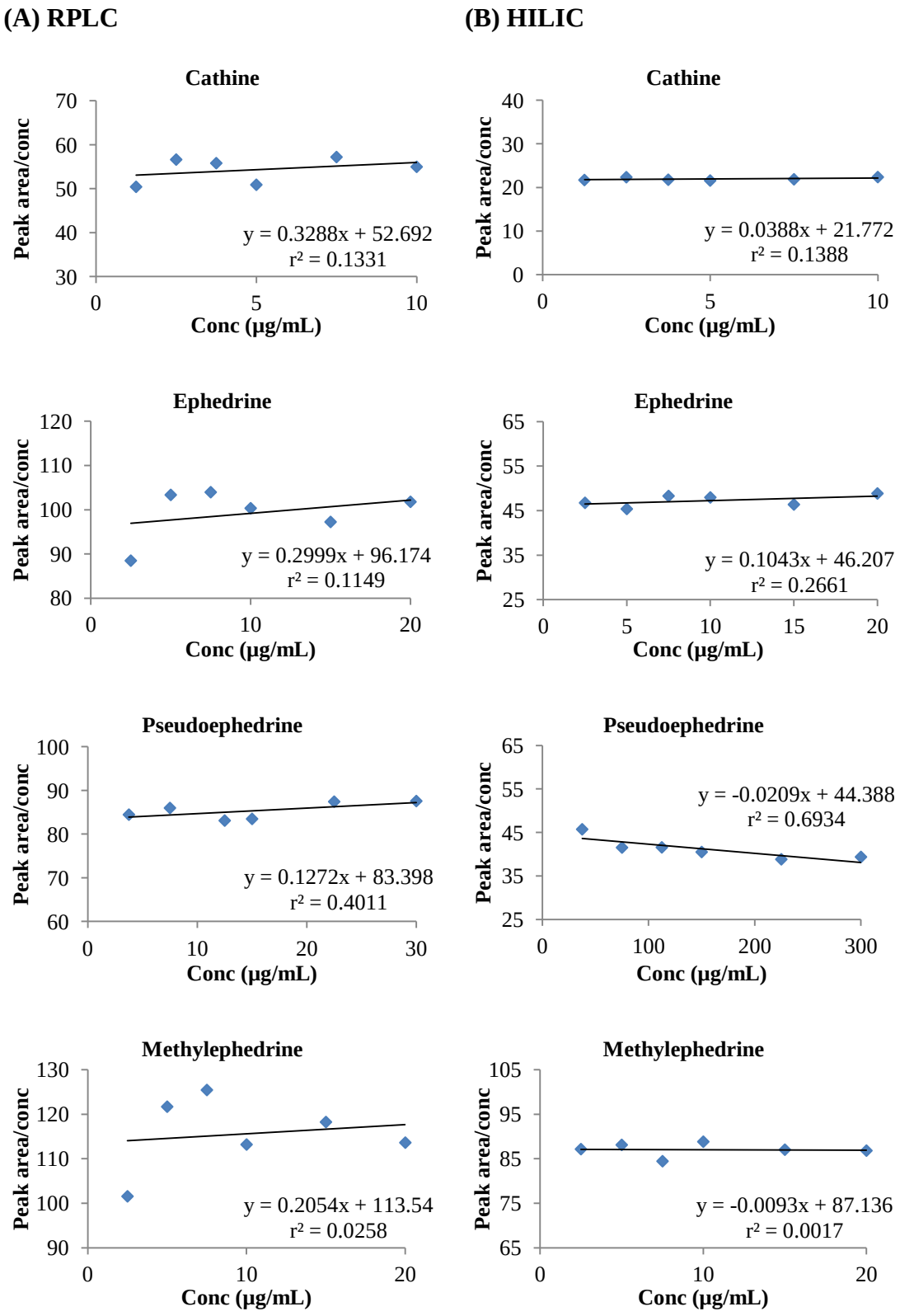
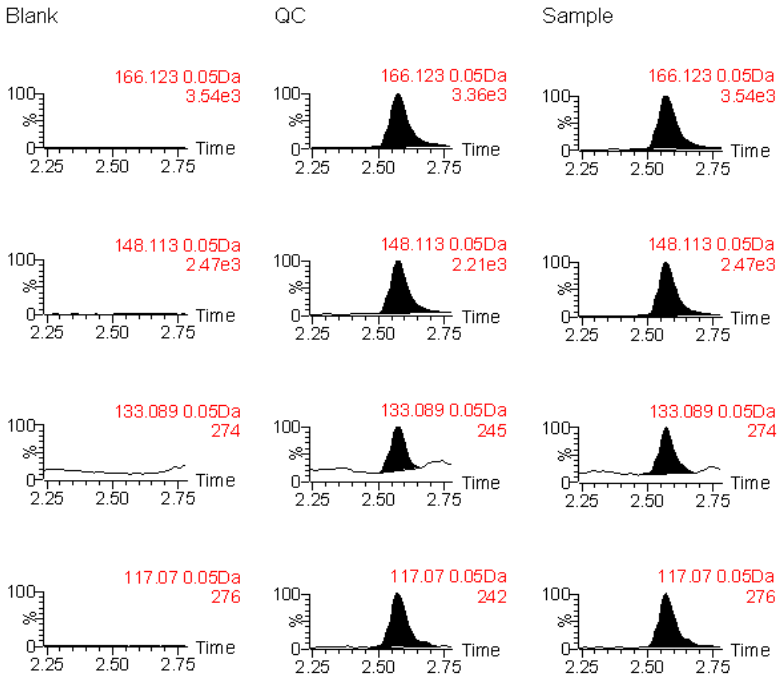


Figure 4.10. Response factor plots of peak area/concentration versus concentration for RPLC (A) and HILIC(B).

4.3.7.7 Application to a real sample

Both methods were applied to the analysis of a suspect ephedrine sample. The results correlate well with one another at 9.34 µg/mL and 9.76 µg/mL with the RPLC pH 10 and HILIC methods, respectively (Figure 4.11).

(A) RPLC pH 10



(B) HILIC pH 5

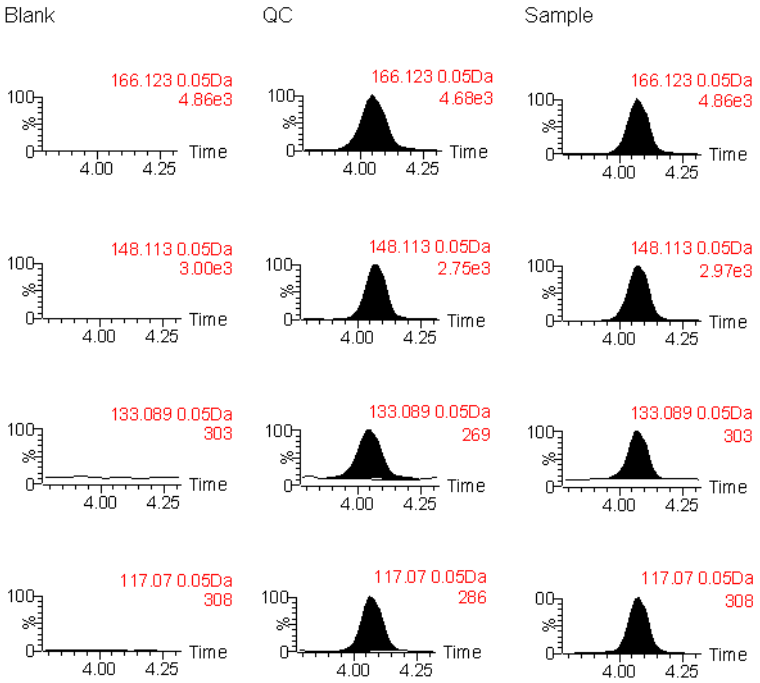


Figure 4.11. Analysis of an ephedrine suspect sample with the RPLC pH 10 method (A) and the HILIC method (B) (0.02 Da mass window). RPLC performed on an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm column operated under gradient conditions at 0.6 mL/min and 45 °C. HILIC separation performed on an Acquity BEH HILIC 1.7 µm, 100 x 2.1 mm column operated under isocratic conditions at 0.5 mL/min and 50 °C.

4.4 Conclusions

A new HILIC-QTOF-MS method has been developed and validated as a complementary alternative to traditional RPLC approaches for the separation and quantification of ephedrines. A comparison was presented between high pH RPLC and HILIC methodologies for the quantitative analysis of hydrophilic basic analytes, exemplified by the ephedrines for doping control analysis. Both approaches demonstrate sufficient retention and resolution of the critical pairs, but HILIC mode offers improvements in peak shape and enhanced sensitivity with ESI-MS detection. In addition, the greater dynamic range offered by HILIC in this application illustrates the potential to analyse samples where components are present over a wide range of concentrations without saturating the stationary phase. With the dilution of pseudoephedrine, no significant differences were noted for linearity, accuracy and precision between the two approaches. Both methods are amenable to a quick and simple dilution and injection of urine samples, omitting time consuming and laborious sample preparation treatment. Ionisation variability from possible matrix interference was negligible for both sets of conditions, although slightly more variation was observed in HILIC mode. One major drawback of HILIC is the expensive solvent system required, and therefore future efforts may be made in fostering alternative organic components or the use of micro scale separations for reduced mobile phase consumption.

Despite initial challenges with retention time reproducibility, HILIC illustrates a robust and reliable alternative for the accurate quantification of hydrophilic bases. Quantification using UHPLC-QTOF mass spectrometry provided linear, accurate and precise results with high sensitivity sufficient for the determination of cathine at

5 µg/mL following a 1:90 dilution step. The advantage of HRAMS over traditional triple quadrupole MS/MS systems is that little MS method development time is needed, qualitative and quantitative analysis on the same samples can be acquired in the same analysis and full scan data can be acquired without compromising the number of data points per peak despite the number of analytes detected at one time. Continuous SRM intuitively gives better signal-to-noise ratios, however such instruments can suffer from limited dynamic range, whereas the type of detector used here has the means of attenuating the ion intensity thereby offering an extended dynamic range. Modern QTOF instrumentation offers fast scanning capabilities for use with UHPLC technology at an affordable cost. This medium-resolution type of detector coupled with prior UHPLC separation provides sufficient resolution to eliminate interferences, together with achieving reasonable mass accuracy.

CHAPTER 5

COLUMN SWITCHING FOR THE COMPREHENSIVE ANALYSIS OF POLAR AND NON-POLAR COMPOUNDS

5.1 Introduction

The issues relating to the analysis of polar compounds are apparent in a variety of fields. Small, highly polar compounds have recently come to the forefront in pharmaceutical and biomedical applications, alongside an increased interest in the separation of highly polar metabolites in drug metabolism and pharmacokinetic (DMPK) studies and emerging metabolomics approaches. Consequently, the need to screen samples for a diverse range of unknown analytes is often presented. The separation of polar and non-polar solutes is a challenge, typically requiring a combination of several approaches, including reversed-phase liquid chromatography (RPLC), normal-phase chromatography (NPC), hydrophilic interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE). However, with more and more analytes to separate and the need for fast turnaround times, high-throughput methodologies are becoming essential. A generic approach which encompasses a wide range of physiochemical properties, permitting simultaneous screening in a single run, is therefore highly desirable.

As discussed in previous chapters, the retention and separation of hydrophilic compounds is an ongoing challenge for RPLC. Alternatives, including NPC, ion exchange chromatography (IEC) and ion pair chromatography (IPC), have significant drawbacks which are mainly associated with mobile phase solvents, additives and high salt buffers that are incompatible with mass spectrometric detection, thus restricting their use. Although alleviated to some extent by the development of highly aqueous-stable phases and the advent of HILIC, the issues relating to the analysis of very polar compounds still remain. HILIC has so far

proven to be the most successful phase for the analysis of very polar analytes and has evolved as an effective technique for the analysis of amino acids, proteins, vitamins and compounds of pharmaceutical interest [145, 150, 158, 159]. However, there remains no single solution that provides a separation for a wide variety of compound polarities, particularly when interfacing with MS.

Two-dimensional liquid chromatography (2D-LC) has demonstrated benefits in generating high peak capacities for a wide range of compounds through the use of orthogonal stationary phases or conditions. 2D-LC employs the use of two independent chromatographic systems that are applied to the same sample. Driven by the necessity for greater resolving power with complex samples, 2D-LC has assumed a popular role in fields such as metabolomics and proteomics where large peak capacities are desired. Although multidimensional separation systems have been largely used to characterise peptides and proteins, such workflows have also been applied to the separation of small molecules in complex mixtures [160] and traditional Chinese medicines [161, 162]. In order to achieve orthogonality, phases with different separation mechanisms should be employed in 2D-LC. Commonly employed workflows include the use of RPLC in the second dimension with IEC, strong cation exchange (SCX), anion exchange (AX), size exclusion chromatography (SEC) or RPLC under different operating conditions in the first dimension. Although NPLC-RPLC is highly orthogonal, NP mobile phases are often immiscible with the RP mobile phases, therefore such a combination is rarely reported. RPLC performed in both dimensions by using different columns or mobile phase pH [163, 164] provides large peak capacities but less orthogonality, and can also suffer from mobile phase incompatibility unless an isocratic elution is performed in the first

dimension. Where the drive is to separate extremes of polarity, the most complementary techniques are HILIC and RPLC, which offer high orthogonality and high peak capacity. Although HILIC has the advantage of being MS compatible for application in the second dimension, the highly organic mobile phase of this technique suffers from incompatibility with those employed in RPLC. Although they are miscible, mobile phases employed in HILIC typically comprise 75-95% organic solvent, whereas RPLC separations start with low percentages of organic and increase over the gradient. As a result, it is not easy to couple RPLC and HILIC for comprehensive on-line 2D-LC. Again, dilution would be required with a highly organic solvent for the HILIC mode and analytes must be soluble in a high percentage of organic modifier, which is often difficult when considering highly polar analytes that have limited solubility in acetonitrile. The direct coupling of RPLC with HILIC in series has been demonstrated as one approach to broadening the elution window for the analysis of polar and non-polar pharmaceutical ingredients [165]. Although the chromatography did not suffer from being subjected to two different modes of separation by the serial coupling of these phases, the incompatibility of the two mobile phase systems results in an in-line dilution by infusion of acetonitrile to provide the HILIC mobile phase.

With the need for fast, high-throughput methods, UHPLC has been widely used to improve peak capacity and analysis times in one-dimensional separations. The difficulty with employing UHPLC in 2D workflows is that any interface must also tolerate ultra-high pressures which, until recently, were either unavailable or very expensive. For this reason, few recent studies exploit the application of UHPLC for 2D separations, which are capable of further enhancing resolving power, reducing

elution time and increasing sensitivity [166-169]. One approach for complex impurity analysis is that reported by Huidobro *et al,* who used off-line RP x RP with UHPLC in the second dimension [167]. An alternative approach is reported by Alexander and Ma *et al*. who use a fused-core material as an alternative in the second dimension as an in order to achieve fast separations at ambient temperature without excessive back-pressure for the analysis of pharmaceutical samples [170].

Small molecules with many polar groups have been shown to be well retained on polymeric resins compared with the poor retention exhibited on bonded silica phases. Compared with the bonded phases, polymeric sorbents typically have higher specific surface areas and increased capacity due to the greater percentage of organic material. Frequently used as solid phase extraction (SPE) sorbents or as monolithic materials, strong hydrophobic (SH) resins can illustrate significant retention of highly polar solutes [171, 172]. However, such materials are inherently inefficient and, as such, are not widely used as particulate materials for separation platforms. However, polystyrene-divinylbenzene (PS-DVB) is a comparatively robust polymer material, and is used as a polymeric HPLC packing material. The high degree of cross-linking in this polymer gives it an extremely durable nature and enables it to withstand HPLC pressures and resist swelling when used with organic solvents better than alternative polymeric materials. The surface is highly hydrophobic and is frequently modified to produce ion exchangers, such as sulfonated resins which are useful for the retention of polar organic compounds [173]. There are reports of using these supports in chromatographic columns where they have been shown to operate with “reversed-phase” like retention characteristics when using hydrophilic solvents as mobile phases [174]. The inherent stability over a wide temperature and pH range

is a distinct advantage of these materials, despite being inefficient compared with silica-based packing materials and not as mechanically strong. For this reason, PS-DVB materials are often reserved for monolithic column formats.

In addition, porous graphitic carbon (PGC) has been demonstrated as a viable phase for the retention and separation of polar analytes [49, 175-177], glycans [178] and several pharmaceutically relevant applications [179-182]. Due to its highly hydrophobic nature, PGC behaves as a strong reversed-phase packing material and is suitable for the chromatography of very polar compounds. The stability of PGC renders it resistant to extremes of pH and temperature and, furthermore, has been shown to offer shape selectivity and the potential to separate positional isomers. One disadvantage of such highly hydrophobic material is the difficult desorption of non-polar compounds adsorbed on the surface, limiting the window of potential polarities. The complex mechanisms of interaction have been shown to involve a combination of hydrophobic and electrostatic interactions between polarisable or polarised functional groups in the analyte with graphite [183]. Hennion *et al.* reported that, in the retention of polar analytes, electronic interactions are more important than hydrophobic interactions [184]. According to Knox and Ross, this effect is particularly strong when the stereochemistry of an analyte forces the polar group close to the graphite surface, known as the polar retention effect by graphite [50, 184]. However, PGC has, to date, largely been unexplored in 2D workflows despite the alternative mechanisms of interaction to conventional RPLC phases, resulting in differences in selectivity. Recently, Griffiths *et al*. illustrated the orthogonality of PGC and RPLC for peptide analysis and highlighted the advantages of PGC over SCX in a 2D-LC workflow for proteomic studies [185].

The ultimate goal in this study is to develop a generic, high-throughput method for the simultaneous retention and separation of hydrophilic and hydrophobic solutes within a single chromatographic analysis. The objective is to design a switching system comprising two different stationary phase materials for the fast separation of a diverse range of small molecules, one for the separation of polar analytes and a complementary phase to separate the more non-polar analytes. Despite the disadvantages of polymeric materials, due to the high retentively of PS-DVB, a proprietary column packed with 7 µm particles was evaluated to determine its applicability in retaining and resolving very polar small molecules. The potential of this phase was investigated as a complementary phase to reversed-phase C_{18} material. In addition, the high retentivity of PGC towards polar molecules makes this phase an attractive option for this application and is also evaluated.

Due to pressure constraints, until recently it has been difficult to construct a column switching system where UHPLC can be used in the first dimension, requiring interface valves able to withstand operation at ultra-high pressures (up to 15,000 psi). With the ultra-high pressure switching valves employed here, the use of UHPLC in this column switching design is investigated in order to achieve faster analysis times.

5.2 Experimental

5.2.1 Materials

Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK) and formic acid (99-100 %) was purchased from VWR (Leicestershire, UK). Uracil and guanine were purchased from Fluka (UK). Acetophenone, adenine, aminoimidazole carboxamide ribonucleotide (AICAR), atenolol, benzoic acid, caffeine, clobestalol, cytidine, cytosine, diclofenac, ethacrynic acid, flumethasone, formoterol, hexanophenone, oxycodone, p-hydroxyamphetamine, salmeterol, sotalol, terbutaline, testolactone, testosterone, thymine and uridine were obtained from Sigma-Aldrich (Gillingham, UK). Water was purified by an ultra-pure water system (Milli-Q, UK).

Stock solutions were prepared at a concentration of 1 mg/mL in methanol water for all compounds except cytosine, cytidine, uracil and uridine, which were prepared at 1 mg/mL in water, and adenine, guanine and thymine, which were prepared at 1 mg/mL in 75:25 v/v CH_3CN:CH_3OH and stored at 4 °C. Standard working solutions were prepared by diluting stock solutions to the desired concentration with mobile phase (95:5 v/v H_20:CH_3CN with 0.1 % formic acid).

5.2.2 LC conditions

Separations were carried out on a Jasco X-LC gradient pumping system equipped with a JASCO X-LC autosampler (JASCO Corporation, Tokyo, Japan). Two six-port ultra high pressure valves were used on the switching system (Rheodyne, CA, USA and Valco Instruments Co., Houston, TX) each operated by an automatic actuator. Columns evaluated in this study included: a proprietary polystyrene-divinylbenzene

(PS-DVB) 7 µm, 100 x 2.1 mm (PolymerLabs, UK), a material which was facing ongoing evaluation in the research laboratories of Dr. Boughtflower at GSK, Phenomenex C_{18} 5 µm, 50 x 2.0 mm (Phenomenex), XBridge C_{18} 3.5 µm, 50 x 2.1 mm (Waters), Acquity BEH C_{18} 1.7 µm 50 x 2.1 mm (Waters), Acquity BEH C_{18} 1.7 µm 50 x 3.0 mm (Waters), and Hypercarb 5 µm, 100 x 4.6 mm (Thermo). The mobile phase consisted of 0.1 % formic acid in purified water (A) and 0.1 % formic acid in acetonitrile (B). The injection volume was 10 µL and the needle wash was methanol. Separations were performed at ambient temperature.

Analytes were detected by ultra-violet (UV) detection using an Applied Biosystems 783A Programmable Absorbance Detector equipped with a micro flow cell (2.4 µL). Analytes were detected at a wavelength of 254 nm with a rise time of 200 msec. Instrument control and data acquisition were performed by ChromPass software (JASCO Corporation, Tokyo, Japan). To minimise system volume, 0.0005" i.d. tubing was used of the minimum length possible between the valves and the columns and between the column and the detector cell. Figure 5.1 illustrates the configuration of the system.

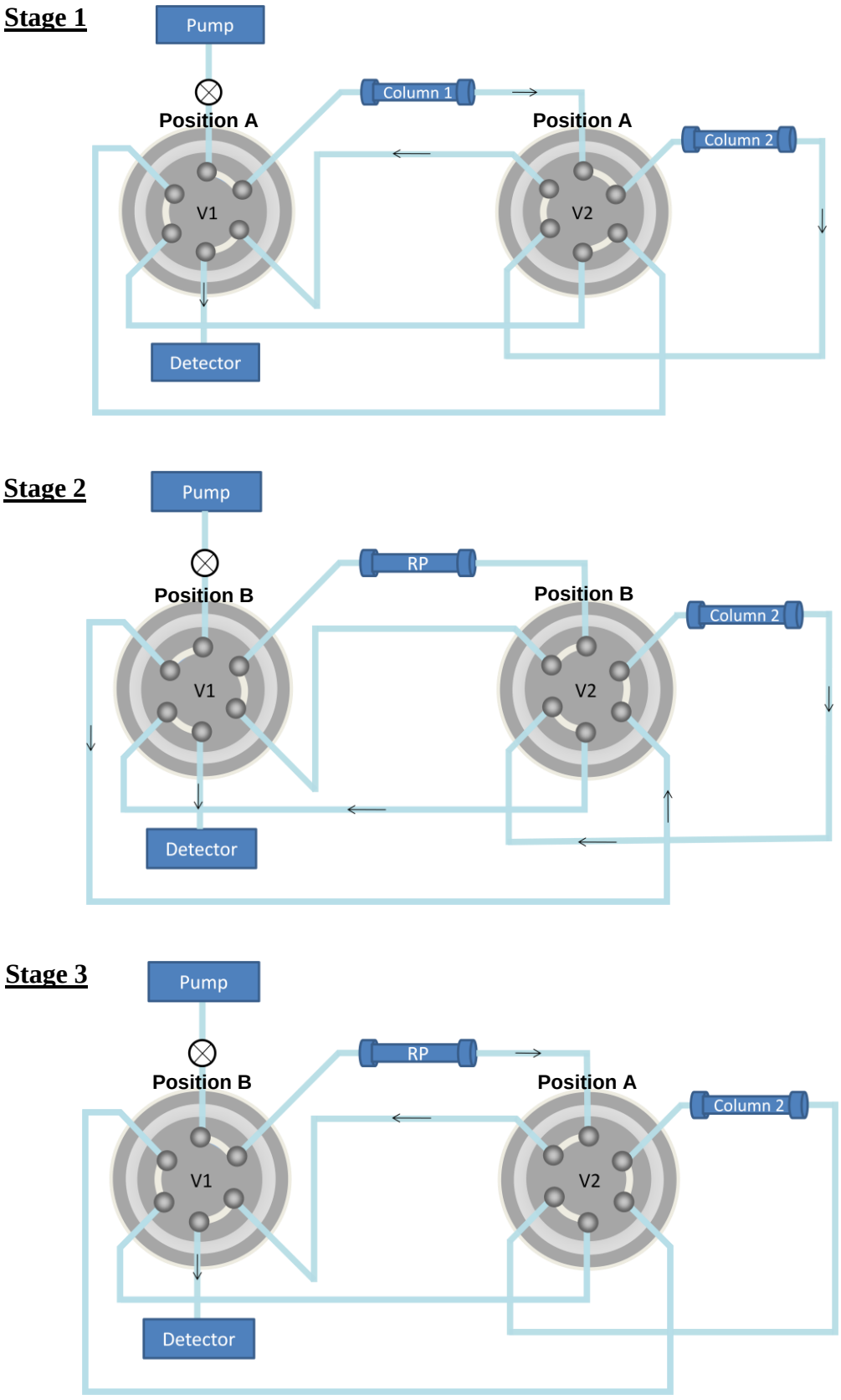


Figure 5.1. Schematic of the instrumental switching design comprising two 6-port ultra high pressure valves (V1 and V2) and their switching positions (Position A and Position B).

The proof-of-principle experiment was performed with nine compounds chosen to cover a wide range of polarities. The optimised conditions employ the use of a BEH C_{18} 1.7 µm, 50 x 3.0 mm as the first column and a Hypercarb 5 µm, 100 x 4.6 mm as the second column. The sample was introduced onto the first column at a flow rate of 0.1 mL/min at 5 % B followed by the gradient profile detailed in Table 5.1. Valves 1 and 2 were switched between positions A and B at the times detailed in Table 5.1 to enable loading, elution from column 1 and 2 and re-equilibration of the whole system.

Table 5.1. Gradient profile and valve switching for method used in the proof-of-concept study.

Time (min)	Flow rate (mL/min)	%B	Valve 1	Valve 2
0.0	0.1	5	Position A	Position A
6.0	0.1	5	Position B	Position B
6.1	0.6	5		
7.0	0.6	5		
17.0	0.6	95		
17.1	0.6	5		
20.0	0.6	5	Position B	Position A
23.0	0.6	95		
23.1	0.6	5		
25.0	0.6	5		
26.0	0.1	5	Position A	Position A
35.0	0.1	5		

To evaluate the applicability of the column switching approach, a mixture of 12 doping agents spanning the polarity range was then studied. The optimised method was based upon that described above, using the same columns and mobile phase compositions. The gradient program and valve switching times are detailed in Table 5.2.

Table 5.2. Gradient profile and valve switching times for analysis of polar and non-polar doping agents.

Time (min)	Flow rate (mL/min)	%B	Valve 1	Valve 2
0.0	0.1	5	Position A	Position A
5.0	0.1	5	Position B	Position B
5.1	0.6	5		
6.0	0.6	5		
9.0	0.6	5		
13.0	0.6	20		
16.0	0.6	95		
16.1	0.6	5		
19.0	0.6	5	Position A	Position B
20.0	0.6	20		
23.0	0.6	95		
24.0	0.6	95		
24.1	0.6	5		
26.0	0.6	5	Position A	Position A
27.0	0.1	5		
34.0	0.1	5		

5.3 Results

5.3.1 Suitability of stationary phase materials

The initial part of this investigation was intended to evaluate the suitability of the proprietary PS-DVB for the retention and separation of polar analytes. For use in this instrumental set-up where UHPLC pressures are desirable, the phase was evaluated for its ability to retain and separate highly polar test probes with suitable efficiency and its ability to withstand the UHPLC pressures without compressing.

5.3.2 Polymeric phase (PS-DVB)

Polar compounds which are difficult to retain by RPLC have previously demonstrated good retention behaviour on highly cross-linked PS-DVB sorbents which display strong hydrophobic characteristics [186]. Very polar nucleobases were used as test probes to evaluate this phase, which was then compared with a hybrid C_{18} material operated under the same conditions. The chromatographic differences towards the same analytes are illustrated in Figure 5.2. The strong retention characteristics of the PS-DVB material, compared with the lack of retention exhibited with the hybrid C_{18} phase, are displayed in Figure 5.3.

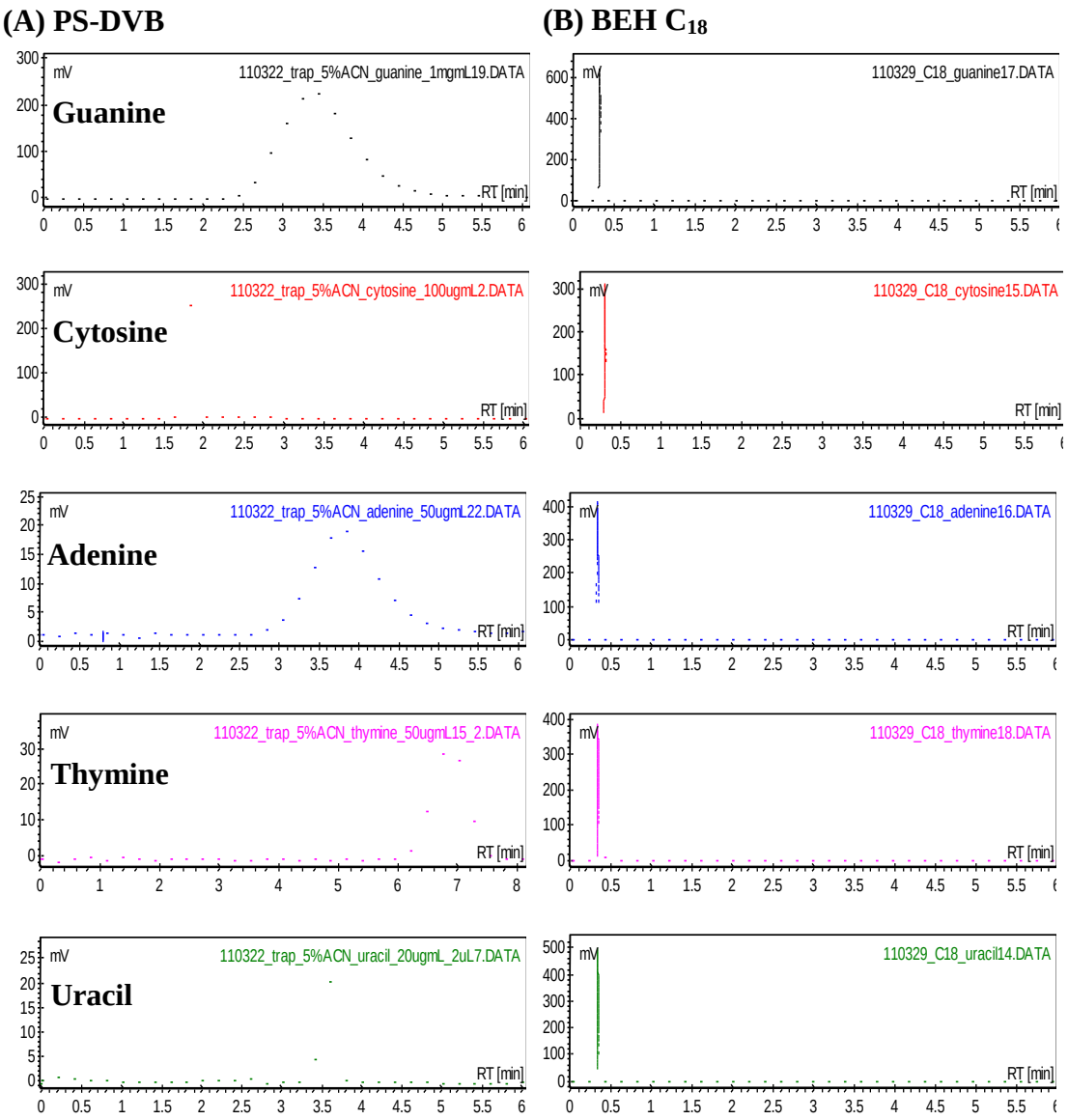


Figure 5.2. Chromatograms illustrating differences in retention characteristics towards nucleobases of the PS-DVB phase (A) and a BEH C_{18} phase (B).
Conditions were isocratic using 95:5 v/v H_20:CH_3CN 0.1 % formic acid.

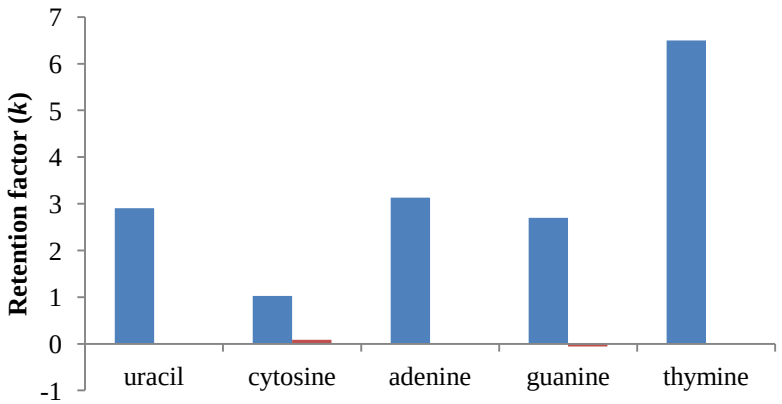


Figure 5.3. Retention factor of polar analytes on the PS-DVB resin (blue) and on C_{18} (red).
Conditions were isocratic using 95:5 v/v H_20:CH_3CN 0.1 % formic acid.

Despite the great potential of this material in retaining highly polar analytes, due to the inherent poor chromatographic efficiency of the 7 µm gel, susceptibility to swelling and shrinkage when running gradient chromatography and inability to withstand high pressures created with the prior UHPLC phase, it was not suitable for this application. This was seen by a gradual decrease in efficiency and distorted peak shapes. On investigating the packed bed, a large void at the head of the column was noted, which confirmed the inability of this phase to withstand the required pressures.

5.3.3 Porous graphitic carbon (PGC)

As a result of the poor physical stability of the PS-DVB phase investigated, PGC (Hypercarb) was evaluated as an alternative due to its previously reported success in the analysis of very polar molecules [49, 175]. Despite the disadvantages in its unpredictable behaviour and lack of efficiency compared with other materials, such as HILIC, the ability to retain polar analytes while operating under reversed-phase mobile phase conditions is a distinct advantage. Since good retention and peak shapes were obtained for the nucleobases on Hypercarb, three of these analytes were chosen as test probes for a proof-of-principle investigation to establish the feasibility of combining this with the second C_{18} column for a comprehensive switching system for the analysis of a wide range of polarities.

5.3.4 System design

The column switching system was configured to incorporate two complementary stationary phases for the comprehensive analysis of polar and non-polar analytes in a

single run. The set-up was designed to operate with compatible mobile phases for facile switching between the two stationary phase components. The resulting system is a simple set-up comprising two ultra-high pressure 6-port switching values, one binary gradient pumping system and a single detector. Whereas typical orthogonal 2D-LC configurations require two pumping systems for two different solvent systems, in this instance both stationary phases were selected for their ability to operate under reversed-phase conditions, hence only necessitating one UHPLC pump.

Figure 5.1 shows the schematic of the instrumental design, where columns 1 and 2 were chosen for their ability to retain and separate non-polar and polar analytes, respectively. Column 1 is a C_{18} phase for the analysis of less polar compounds, while Hypercarb was used as column 2 for its potential to analyse very polar compounds under reversed-phase conditions and the ability to withstand the fast flow rates and UHPLC pressure required.

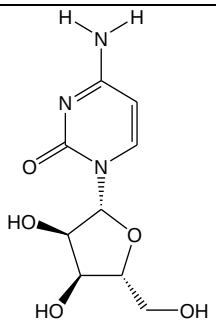
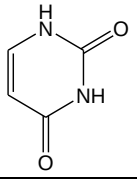
Figure 5.1 illustrates the workflow with the three switching steps required. In Stage 1, both valve 1 (V1) and valve 2 (V2) are in position A, allowing mobile phase to flow through column 1 (BEH C_{18}) and column 2 (Hypercarb). In Stage 1, the sample is loaded with 5 % B where the polar analytes not retained by the C_{18} ligands and are eluted from column 1 and onto column 2, while the more non-polar compounds are trapped on column 1. In Stage 2, both V1 and V2 are switched to position B, allowing mobile phase to flow through column 2 only. In this stage, a gradient programme is employed to elute the polar analytes from column 2, before returning to the starting composition for a re-equilibration step. In Stage 3, V1 is

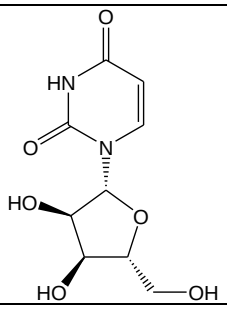
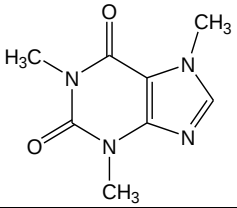
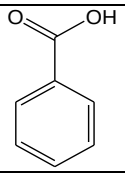
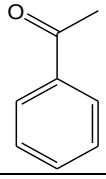
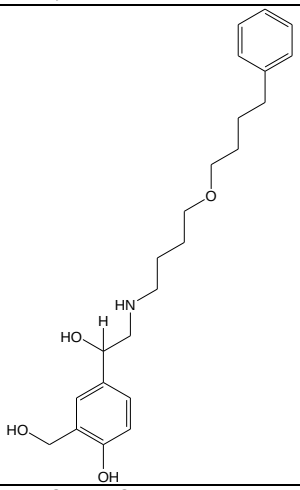
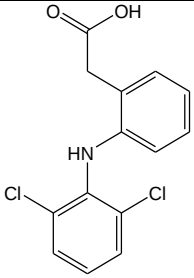
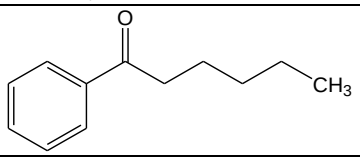
then switched back to position A, allowing flow through column 1 only. This stage therefore allows elution of the non-polar analytes from column 1 under gradient conditions, followed by a brief re-equilibration. The positions as in Stage 1 are then resumed for re-equilibration of the whole system before injection of the next sample.

5.3.5 Proof-of-principle

The proof-of-principle investigation was performed using a test mixture of analytes covering a wide range of polarities, comprising acids, bases and neutral analytes, as detailed in Table 5.3. Of the analytes investigated, the criteria for separation on the Hypercarb phase was a $k < 2$ on the C_{18} phase. The separation of the polar analytes (cytidine, uracil and uridine) and the more non-polar analytes (caffeine, benzoic acid, acetophenone, salmeterol, diclofenac, hexanophenone) were developed separately on the Hypercarb phase and C_{18} phase respectively, as shown in Figure 5.4.

Table 5.3. Chemical structures and log*P* values for the analytes considered in the proof-of-concept study.

Compound	Structure	Log*P**
Cytidine		-2.24
Uracil		-1.09

Uridine		-2.28
Caffeine		-0.8
Benzoic acid		1.86
Acetophenone		1.35
Salmeterol		3.35
Diclofenac		4.12
Hexanophenone		3.25

* LogP values were obtained from ChemSpider.

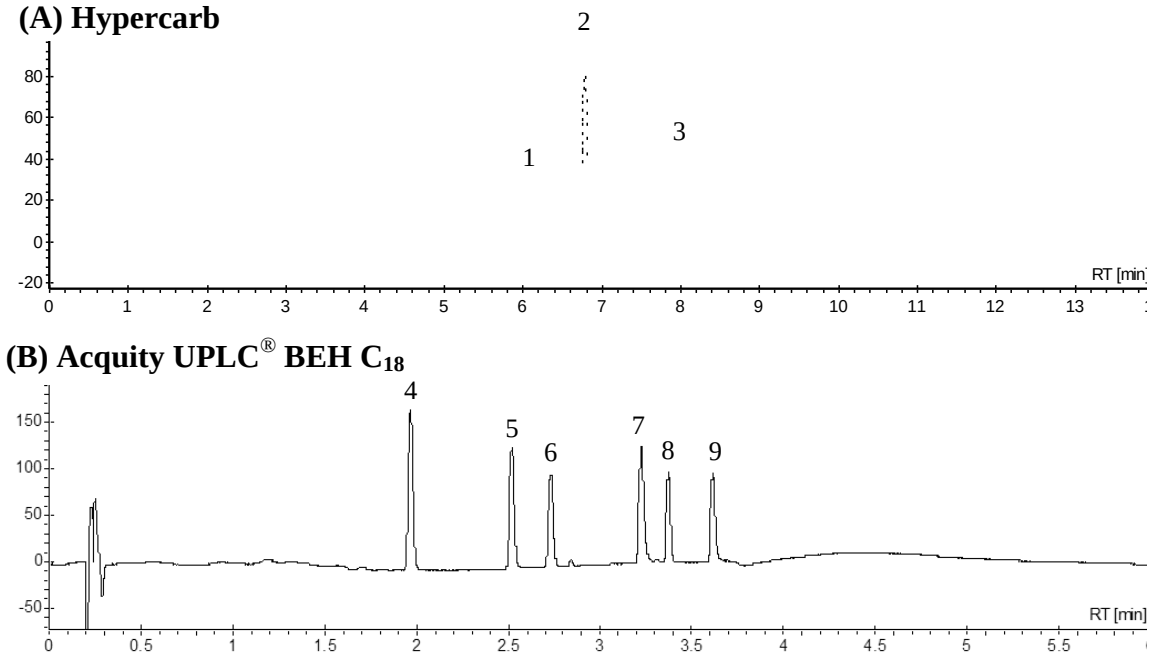


Figure 5.4. Separation of polar analytes on Hypercarb (A) and less polar compounds on C_{18} (B). Cytidine (1), uracil (2) and uridine (3) caffeine (4), benzoic acid (5), acetophenone (6), salmeterol (7), diclofenac (8) and hexanophenone (9). The Hypercarb 5 µm, 100 x 4.6 mm column (A) and Acquity BEH C18 1.7 um, 50 x 2.1 mm column were operated under isocratic using 95:5 v/v H_20:CH_3CN 0.1 % formic acid.

5.3.6 Influence of valve switching on the separation

Having developed the separation of each set of analytes on the two phases independently, the next challenge was to couple them together with the switching interface for analysis of the complete mixture in one injection. An example of the gradient profile is shown in

Figure 5.5. The sample was loaded with 5 % B onto column 1 (C_{18}), and both valves switched after 1 min, by which time the polar analytes ($k < 2$) had eluted onto column 2 (Hypercarb). The first gradient therefore relates to the separation of the polar analytes on the Hypercarb column before V1 is switched back to position A to allow the less polar analytes to be eluted from the C_{18} phase and separated during the second gradient. In the first instance, the polar analytes were injected alone with a zero volume connector in place of column 1 and the Hypercarb column as column 2

(pressure 1117 psi with 5 % B at 0.6 mL/min). This was performed to ensure that the chromatography was not affected by the valve switching. Chromatograms illustrating a blank injection and the polar test mix injected are shown in Figure 5.6. Injection of mobile phase shows that the spikes seen at 1, 14 and 17 min correspond to the valves switching, causing a fluctuation in the baseline of the absorbance detector. Figure 5.6 (B) illustrates that the chromatography of the polar compounds is not compromised by switching the valves, with each analyte showing the same retention time as in Figure 5.4 (A).

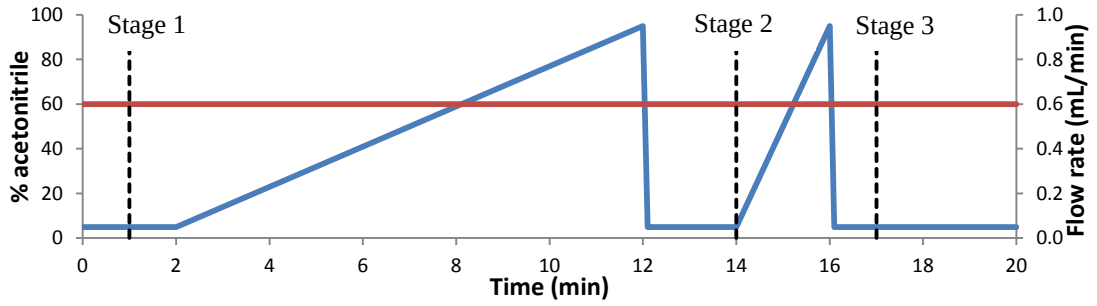


Figure 5.5. Elution profile of the column switching system.
The two gradients (—), flow rate (—) and valve switching times (⋯) are shown.

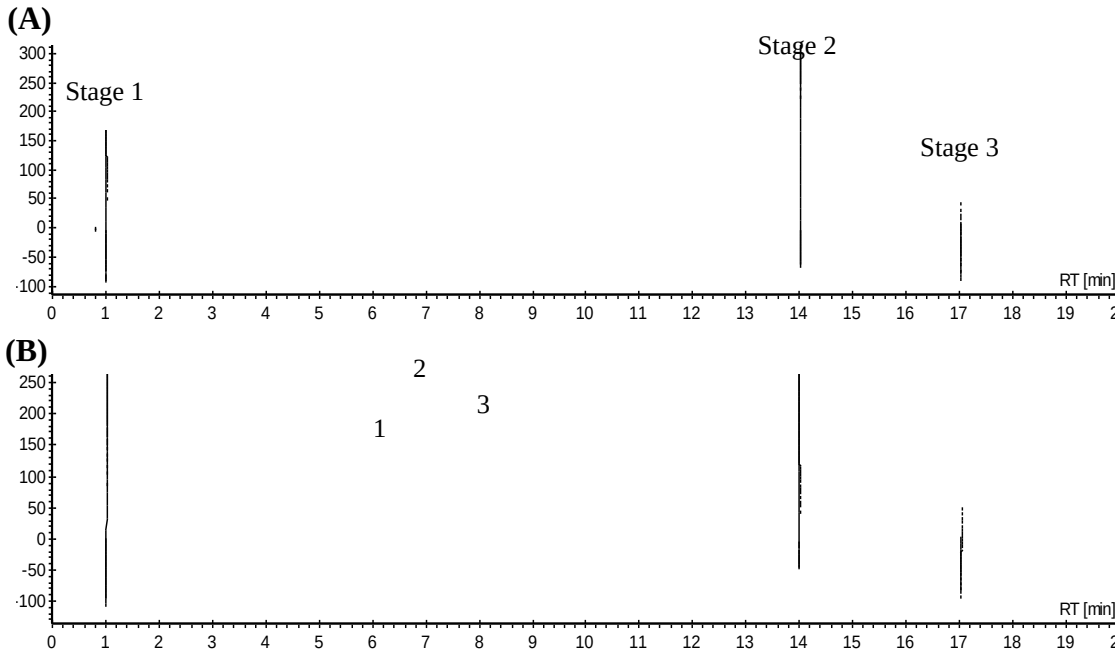


Figure 5.6. Profile obtained with injection of mobile phase with valve switching (A) and the effect of switching valves on separation profile of the polar compounds (B).

For the separation of the polar compounds, cytidine (1), uracil (2) and uridine (3) a zero volume connector in placed in column position 1 (B). A Hypercarb 5 µm, 100 x 4.6 mm was used as column 2.

5.3.7 Influence of pressure and flow rate on the separation profile

Since no change in the retention times of the polar analytes were established with the instrumental set-up using a zero volume connector as column 1, this was replaced with a UPLC® BEH C_{18} 1.7 µm, 50 x 2.1 mm i.d. column to enable the inclusion and separation of the more non-polar analytes in the test mixture. As no retention of the polar analytes was noted on the C_{18} phase, the results were expected to be similar to those generated using the zero volume connector. However, a marked difference in the retention of the polar analytes was experienced when the UHPLC C_{18} column was employed, Figure 5.7 (A).

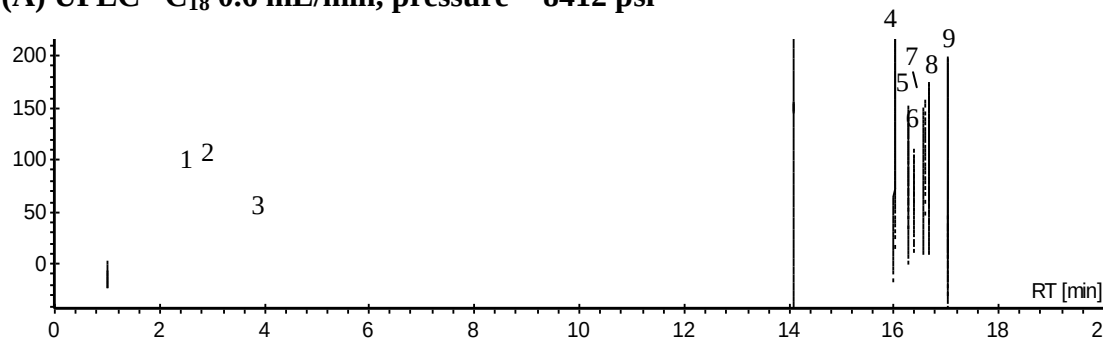
Since there is no retention of the polar analytes with the C_{18} phase, contribution from the column chemistry can be eliminated as a cause of this problem. Therefore, two hypotheses were proposed as contributing to this retention shift – either lack of column re-equilibration, which would lead to a shift in retention and affect the early eluting compounds to a greater extent, or the large pressure difference between the two columns as the configuration was switched from Stage 1 to Stage 2 due to contribution by the sub-2 µm particle UHPLC column. It should be noted that the shift in retention of the polar analytes was only experienced with the UHPLC column when valve switching was introduced. For instance, when the UHPLC column and the Hypercarb column were coupled together in series to analyse the polar mix, the same chromatography was gained as in Figure 5.6 (B). This suggests that it is not the high back-pressure alone that is responsible for the retention shift,

but the large difference in back-pressure when the valves are switched to divert the flow through the Hypercarb phase only, bypassing the UHPLC column.

Poor re-equilibration was investigated as a potential cause, which could feasibly be confounded in this set-up with the extra-column volume, valve switching and two columns. Increase in re-equilibration times for each column and the overall starting set-up were investigated, but increasing re-equilibration time was shown to have no effect on the shift in retention, which remained constant.

Consequently, the sub-2µm column was substituted with a larger particle size column (5 µm) to determine whether the large difference in column back-pressure between the two columns was contributing to the shift in retention. The results noted when a 5 µm HPLC column was used can be seen in Figure 5.7 (B).

(A) UPLC® C_{18} 0.6 mL/min, pressure = 8412 psi



(B) HPLC C_{18} 0.6 mL/min, pressure = 1914 psi

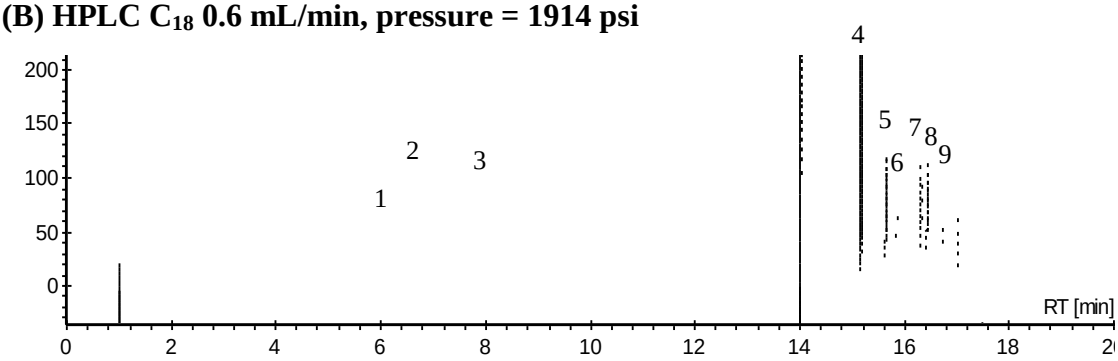


Figure 5.7. Separation profile of polar compounds with a UPLC® BEH C_{18} 1.7 µm, 50 x 2.1 mm i.d. (A) and a HPLC Phenomenex C_{18} 5 µm, 50 x 2.0 mm i.d. (B) as column 1. Cytidine (1), uracil (2) and uridine (3) caffeine (4), benzoic acid (5), acetophenone (6), salmeterol (7), diclofenac (8) and hexanophenone (9). Separation performed using a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

It therefore appears that the shift in retention is related to the large pressure difference between the two phases upon the first switching event (8412 psi across the UPLC® BEH C_{18} column at the beginning of the analysis, compared with 740 psi across the Hypercarb column, when operated with 5 % B at 0.6 mL/min). This is supported by the fact that there is no difference in the retention profile where there was only a negligible difference in the pressure drop between the phases, for example when a zero volume connector or a HPLC C_{18} 5 µm, 50 x 2.0 mm column were used, Figure 5.7 (B).

The pressure difference between the two phases was further investigated in order to overcome the problem. A variety of C_{18} columns of different particle size were evaluated as potential alternatives to the UHPLC sub-2 µm phase for column 1. The differences in pressure drop across these phases as a function of flow rate, compared with the Hypercarb column, are in shown in Figure 5.8. Intuitively, as particle size decreases there is an increase in the back-pressure generated and therefore there is a greater pressure difference between the smaller C_{18} particle columns and the Hypercarb column. Assuming that the greater the pressure difference, the greater the contribution to the retention shift, each of these phases was investigated at various flow rates with the Hypercarb column to determine the acceptable pressure difference where no retention shift is experienced.

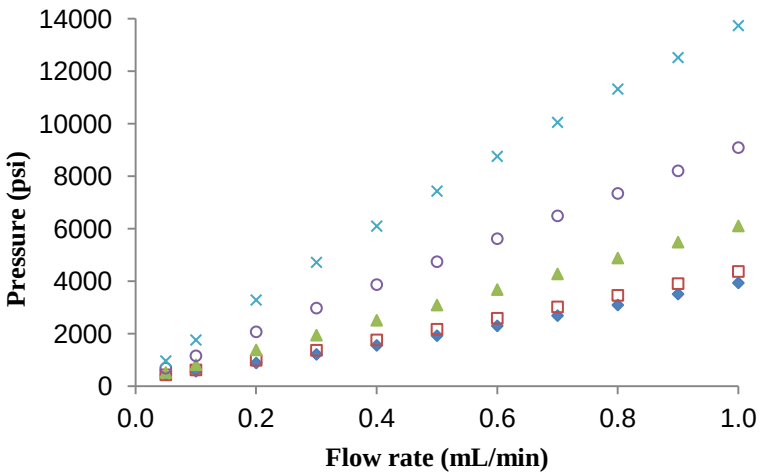


Figure 5.8. Relationship between flow rate and pressure for the different columns studied. Hypercarb 5 µm, 100 x 4.6 mm (♦), Phenomenex C_{18} 5 µm, 50 x 2.0 mm (□), XBridge C_{18} 3.5 µm, 50 x 2.1 mm (▲), BEH C_{18} 1.7 µm, 50 x 3.0 mm (○), BEH C_{18} 1.7 µm, 50 x 2.1 mm (x).

The effect of flow rate and system pressure on the separation profile using a HPLC 3.5 µm column is show in Figure 5.9. These chromatograms illustrate the effect of reducing flow rate, and therefore back-pressure, on the retention shift of the polar analytes (1-3). At 0.3 mL/min, a sufficiently low back-pressure is achieved for repeatable retention times of the polar analytes.

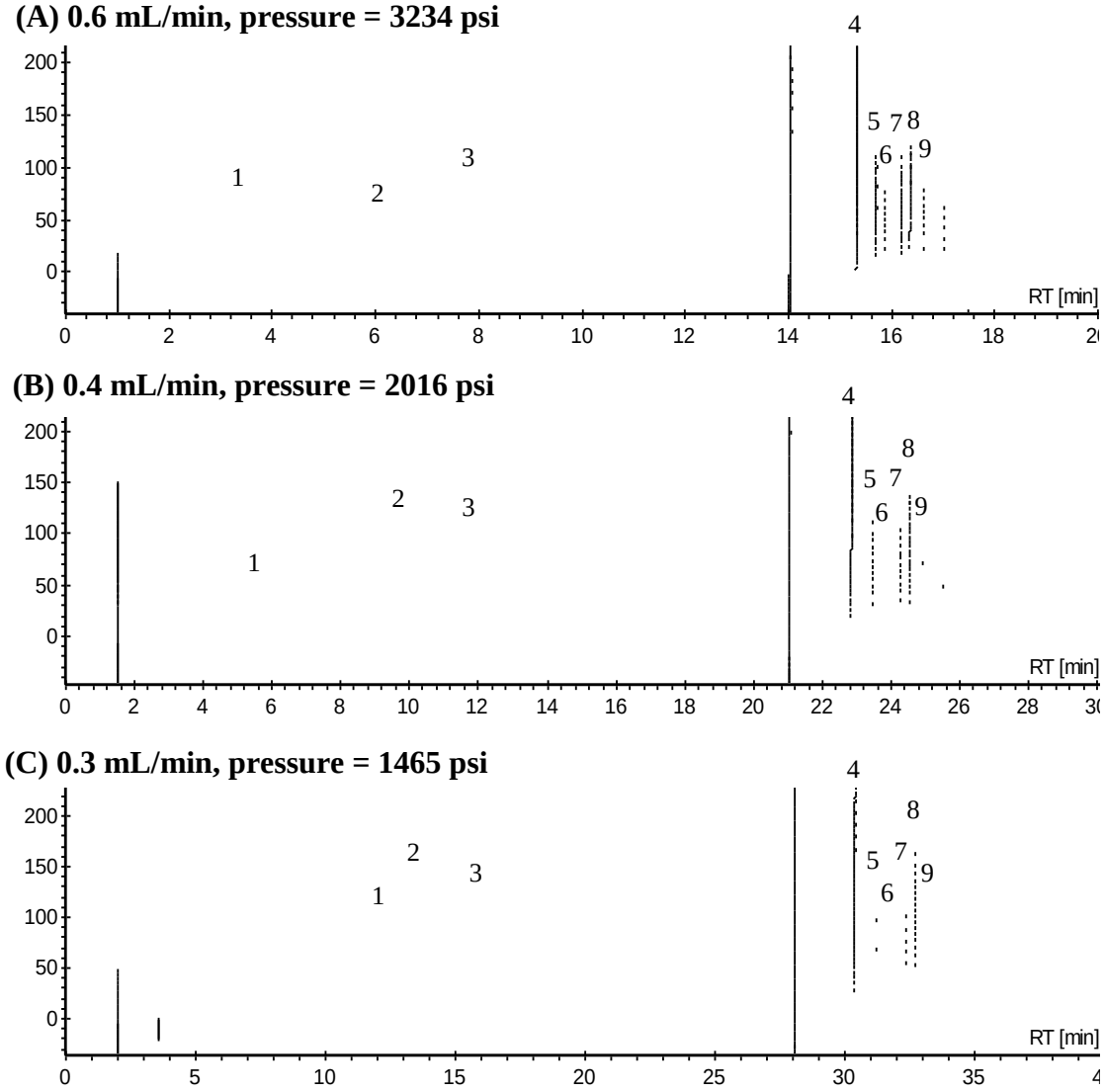


Figure 5.9. Separation profile using 50 x 2.1 mm XBridge C_{18} 3.5 µm operated at 0.6 mL/min (A), 0.4 mL/min (B) and 0.3 mL/min (C).
Compounds numbered as above. Separation performed using an XBridge C_{18} 3.5 µm, 50 x 2.1 mm as column 1 and a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

The same process was repeated with the 1.7 µm UPLC® column and the results are demonstrated in Figure 5.10. Using this column, even a flow rate of 0.1 mL/min still generates a back-pressure too high to prevent repeatable retention of the polar analytes. This column was therefore deemed unsuitable as column 1 in this instrumental configuration.

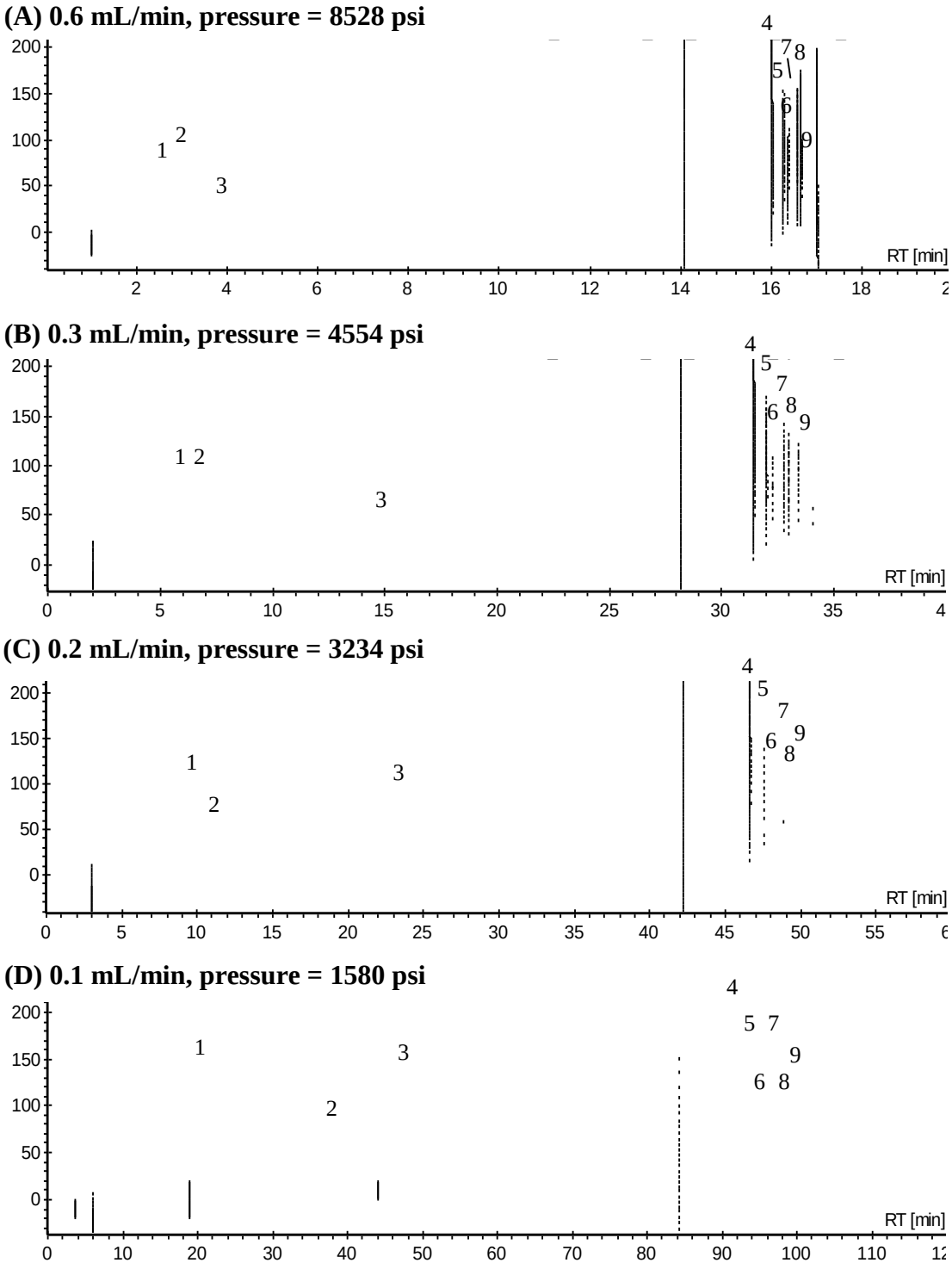


Figure 5.10. Separation profile using 50 x 2.1 mm BEH C_{18} 1.7 µm operated at 0.6 mL/min (A), 0.3 mL/min (B), 0.2 mL/min (C) and 0.1 mL/min (D).
Compounds numbered as above. Separation performed using an Acquity BEH C_{18} 1.7 µm, 50 x 2.1 mm as column 1 and a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

However, the benefits of sub-2 µm particles in increased peak capacity and reduced analysis time for this application are of great significance, hence the use of sub-2 µm

particles packed into a different column format in order to generate lower back-pressures was investigated. A UPLC® BEH C_{18} 1.7 µm 50 x 3.0 mm i.d. column operated under the same conditions as the 2.1 mm variant demonstrates a lower back-pressure at a particular flow rate (Figure 5.8). The chromatograms in Figure 5.11 illustrate the separation profile of the test mix and valve switching method with varying flow rates. At 0.2 mL/min, the back-pressure generated creates too large a pressure difference between the Hypercarb phase when the first valve switching event occurs (1653 psi across the column 1 compared with 740 psi across column 2), resulting in the familiar retention shift of the polar analytes. However, at 0.1 mL/min, the lower back-pressure generated across column 1 (899 psi) is sufficiently low not to cause a large disturbance between the two stages, as shown in Figure 5.11 (B).

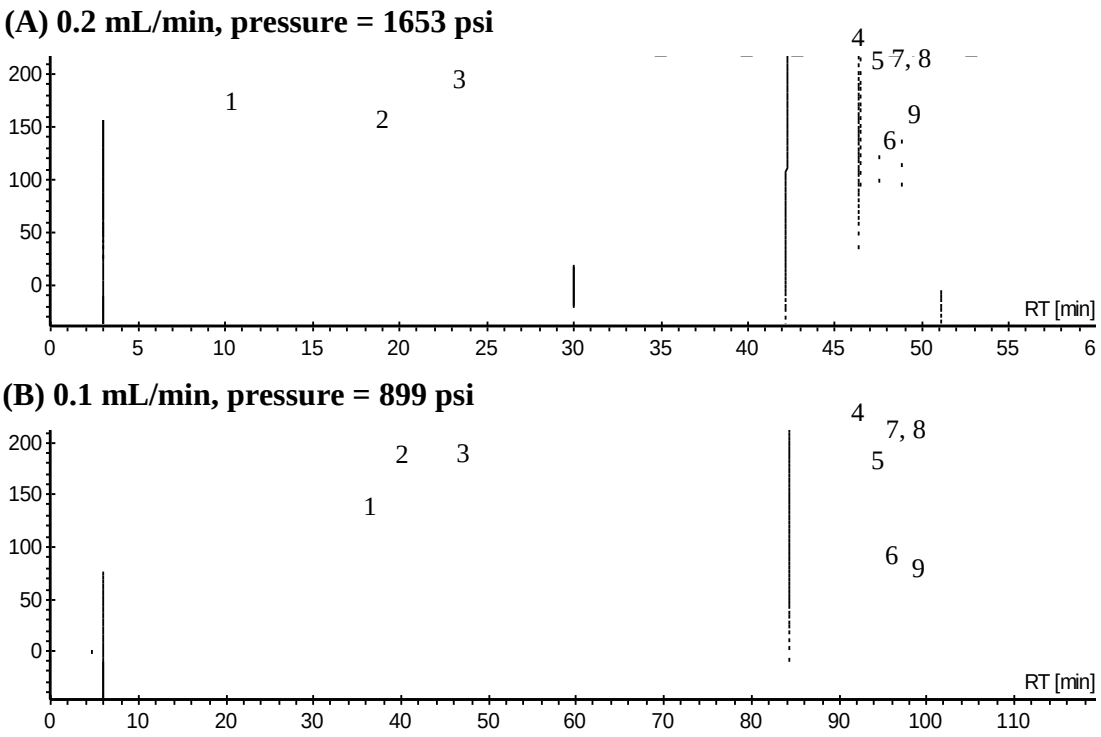


Figure 5.11. Separation profile using 50 x 3.0 mm BEH C_{18} 1.7 µm operated at 0.2 mL/min (A) and 0.1 mL/min (B).
Compounds numbered as above. Separation performed using an Acquity BEH C_{18} 1.7 µm, 50 x 3.0 mm as column 1 and a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

5.3.8 Method optimisation

From the previous results it can be deduced that a large and instant pressure drop when the valves switch between Stage 1 and Stage 2 is detrimental to the separation profile of the polar analytes eluted from column 2. This can be overcome by using a suitable phase and appropriate flow rate so as to reduce the difference in pressure across the two columns as much as possible. Therefore, with the pressure difference in mind, but also considering the long elution time required if a low flow rate was used, the analytes were loaded onto the first column at 0.1 mL/min, and then after the switching of the values the flow rate was increased to 0.6 mL/min for faster analysis. At the end of the run, a flow rate of 0.1 mL/min was resumed for pressure re-equilibration before the next injection, following the profile shown in Figure 5.12. The chromatogram corresponding to this optimised method is depicted in Figure 5.13.

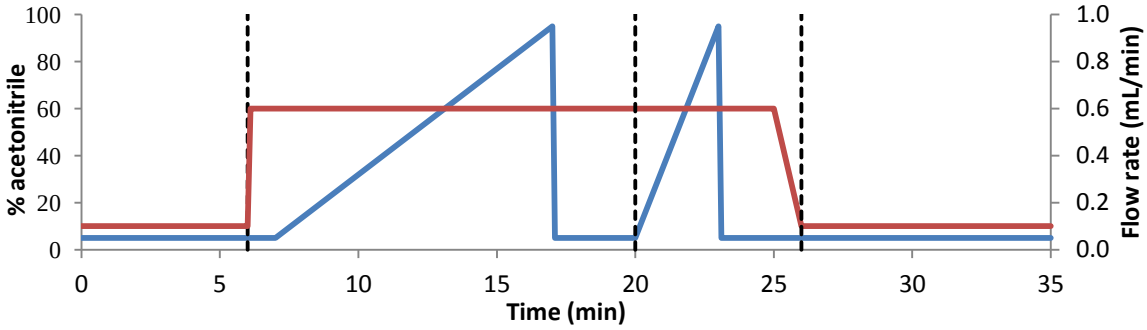


Figure 5.12. Elution profile of the final separation.
The two gradients (—), flow rate (—) and valve switching times (··).

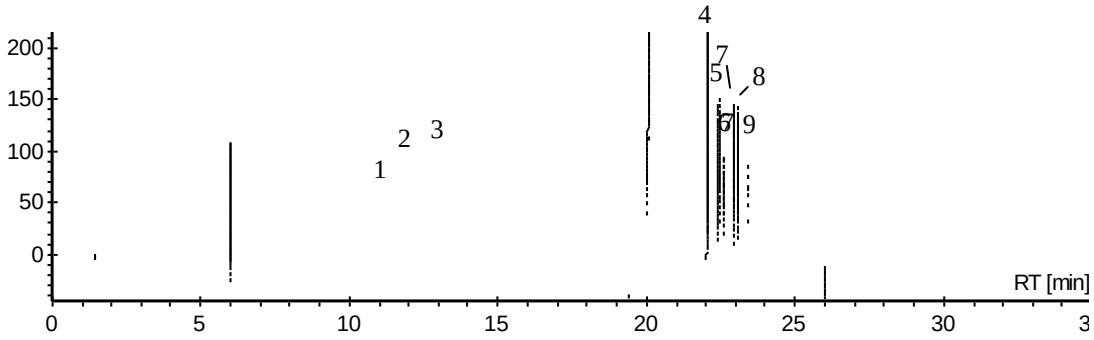


Figure 5.13. Final separation with low loading at a flow rate.
Samples loaded at 0.1 mL/min for 6 minutes before increasing to 0.6 mL/min for elution. Compounds numbered as above. Separation performed using an Acquity BEH C_{18} 1.7 µm, 50 x 3.0 mm as column 1 and a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

5.3.9 Reproducibility of separation

As discussed in previous chapters, a stable system must be ensured to provide valid, robust data. Instability is reflected in shifts in retention time, therefore the reproducibility of the switching method was evaluated over 10 repeat injections. Overlaid chromatograms illustrate the good reproducibility (Figure 5.14), with retention times showing less than 0.06 % RSD (n = 10) (Table 5.4).

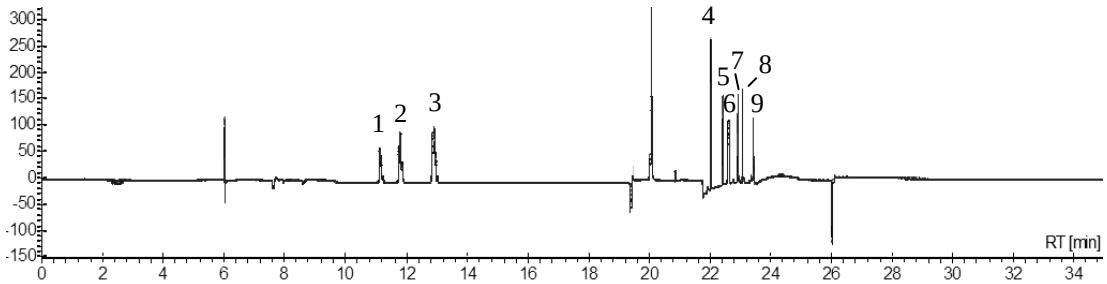


Figure 5.14. Overlaid chromatograms illustrating good reproducibility of the final switching method (n = 10).
Compounds numbered as above. Separation performed using an Acquity BEH C_{18} 1.7 µm, 50 x 3.0 mm as column 1 and a Hypercarb 5 µm, 100 x 4.6 mm as column 2.

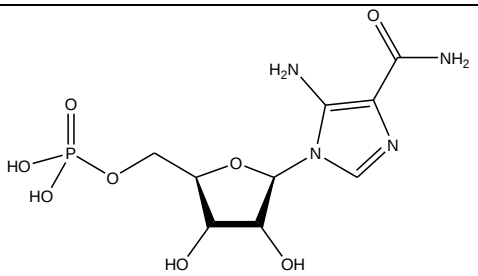
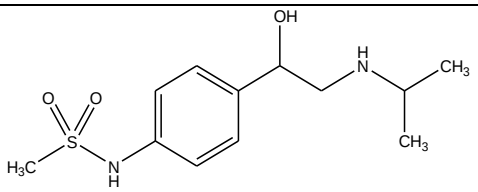
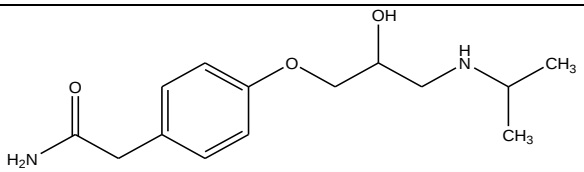
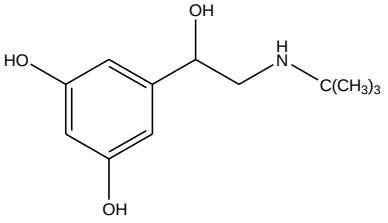
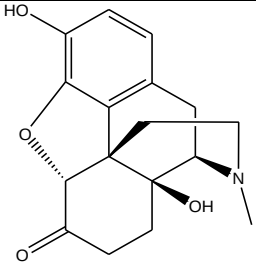
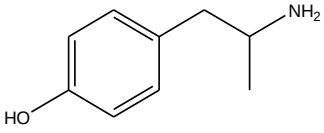
Table 5.4. Retention times of analytes used for proof-of-principle and %RSD values (n = 10).

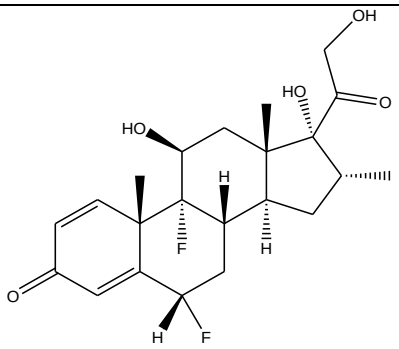
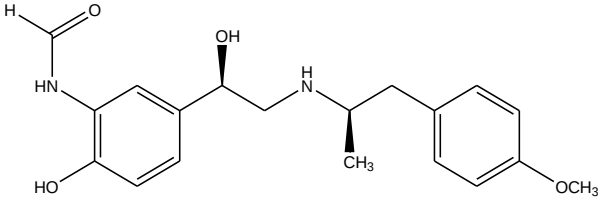
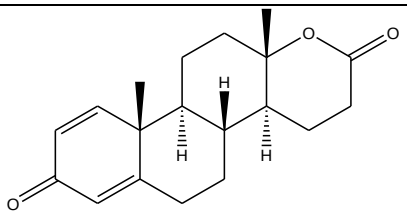
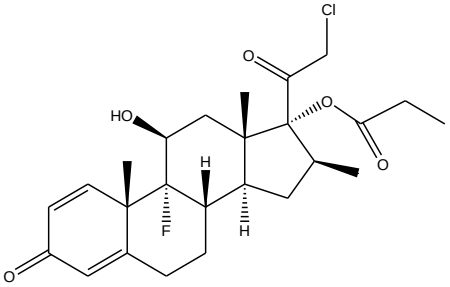
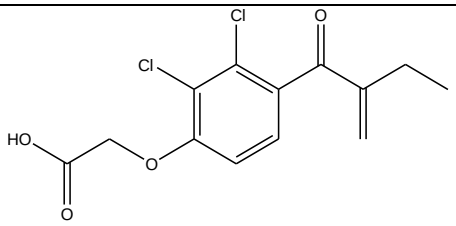
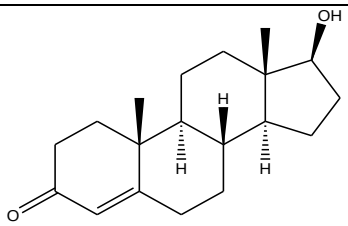
Analyte	Ave t_R (min)	Standard deviation	% RSD
Cytidine	11.1	0.007	0.06
Uracil	11.8	0.007	0.06
Uridine	12.9	0.007	0.06
Caffeine	22.0	0.005	0.02
Benzoic acid	22.4	0.005	0.02
Acetophenone	22.6	0.005	0.02
Salmeterol	22.9	0.005	0.02
Diclofenac	23.1	0.005	0.02
Hexanophenone	23.4	0.005	0.02

5.3.10 Application to doping control screening

Doping control analysis is just one example of where there is a real need to separate a wide range of polarities in a single sample while achieving high analytical throughput. Additionally, it is necessary to gain sufficient retention of highly polar analytes to prevent co-elution with the noisy solvent front which can result in significant ion suppression. Therefore, a mix of 12 compounds screened in doping control analysis covering a range of polarities is analysed here with the column switching technique described previously. Sufficient retention can be empirically described as $k > 2$, therefore an analyte which did not meet this criterion when analysed on a C_{18} stationary phase material with a generic 5-95 % B linear gradient, was considered in the highly polar fraction for analysis on column 2 (Hypercarb). The mixture of test compounds considered is given in Table 5.5, together with the chemical structures and log*P* values to highlight their relative polarities.

Table 5.5. Chemical structures and log*P* values of the doping agents considered as the test probes in this study.

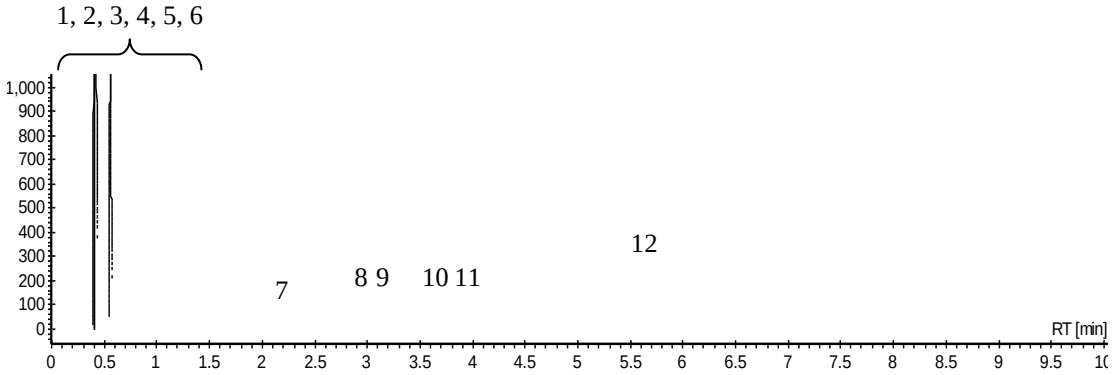
Compound	**Structure**	**Log*P****
AICAR		-2.287
Sotalol		0.24
Atenolol		0.335
Terbutaline		0.696
Oxymorphone		0.898
p-hydroxyamphetamine		1.135

Flumethasone		1.838
Formoterol		2.029
Testolactone		2.225
Clobetasol		2.268
Ethacrynic acid		2.84
Testosterone		3.179

*LogP values were obtained from ChemSpider.

Figure 5.15 (A) illustrates the chromatography of the test mixture which is achieved on a C_{18} phase alone, illustrating no retention or separation of the polar analytes, compared with the retention and separation possible when using the column switching approach with Hypercarb in order to retain and separate the polar analytes (Figure 5.15 (B)). The same solvent system as that employed in the proof-of-principle separation was used for the doping compounds, with a modified gradient. The samples were loaded under the same starting conditions with 5 % B at 0.1 mL/min.

(A) C_{18} only



(B) Column switching with Hypercarb and C_{18}

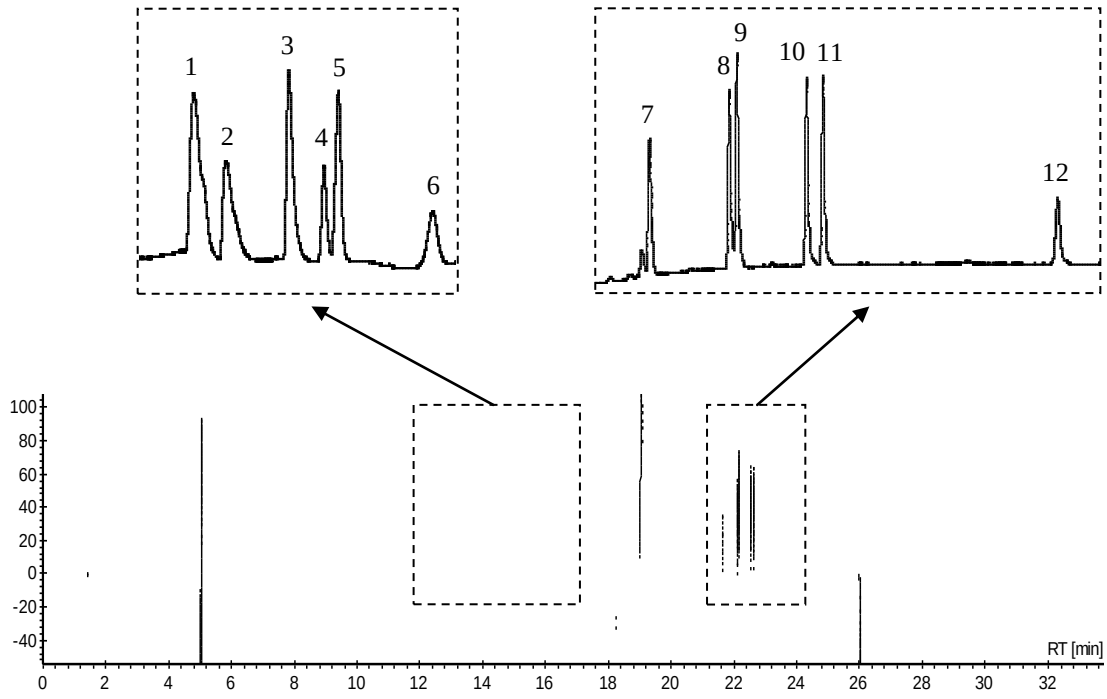


Figure 5.15. Separation of the doping test mix using the column switching approach.
Comparison of a C_{18} phase alone (A) and the column switching approach with Hypercarb and C_{18} as the two complementary phases (B). Terbutaline (1), p-hydroxyamphetamine (2), oxymorphone (3), sotalol (4), atenolol (5), AICAR (6), formoterol (7), testolactone (8), flumethasone (9), clobetasol (10), ethacrynic acid (11), testosterone (12).

5.4 Conclusions

The work presented here highlights some of the challenges facing the comprehensive analysis of compounds with a diverse range of polarities. A switching system enabling a broader window of analysis has been designed and tested with two complementary stationary phases enabling the retention and separation of polar and non-polar compounds. Where resources are limited, it is of primary importance to maximise the use of available instrumentation. This approach allows the analysis of a wide range of compounds in a single analysis with one binary pumping system, a single detector and operated by standard software.

The downsides of such an approach are the large amounts of extra-column volume posed by the tubing required in the plumbing of the system, which negatively influences the potential efficiency gain with sub-2 µm materials. Although Hypercarb is not itself an ideal phase for this application with regard to efficiency, it demonstrates the possibility of coupling together two complementary phases for the analysis of a wide range of polarities in a single run while maintaining MS operation. The advantage of this combination, compared with previous methodologies, is that both phases can be operated under reversed-phase conditions, thereby only necessitating a single mobile phase system with one solvent manager and, as a result, does not involve any in-line sample dilution. Here, efficiency is compromised due to the large particle size of the Hypercarb material (5 µm) and large column dimensions, together with the inherently large extra-column volume contributing to band broadening. However, the principle of using Hypercarb to retain and separate highly polar analytes under reversed-phase conditions is presented. It is important to highlight the possibility of coupling complementary phases to C_{18} without

introducing incompatible mobile phases requiring additional pumping systems and inherent sample dilution. Further work should therefore centre on developing a suitable phase for this application in order to improve efficiency and miniaturisation to minimise extra-column band spreading as much as possible.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions and Future Work

The evolution of LC-MS instrumentation over the last 15 years has significantly impacted upon analytical strategies for targeted screening in bioanalysis. Gas chromatography coupled with mass spectrometry (GC-MS) has previously been considered as being the preferred method for the analysis of small molecular weight analytes. In many areas, including doping control analysis, GC-MS was historically the "gold standard," providing a sensitive and selective technique with good reproducibility, resolution and an inherent ease of coupling with MS detection. However, LC-MS has now become an extremely powerful tool for the high efficiency separation of a wide range of analytes. Increased LC resolution with the use of sub-2 µm particles and the development of UHPLC, together with modern fast scanning mass spectrometers have resulted in LC-MS becoming widely adopted. LC-MS techniques now offer several advantages over GC-MS, including the ability to analyse thermolabile or non-volatile compounds, simple sample preparation with no derivatisation, faster analysis and the ease of directly injecting samples in solution. Consequently, LC-MS has become particularly popular where high-throughput analyses are required with minimal sample pre-treatment to minimise additional sources of variation.

However, the separation of very polar and/or basic compounds remains problematic for LC-MS. The generation of novel stationary phase materials offers certain improvements, although there is still no one simple solution. The importance of pH in RPLC is demonstrated here as a powerful chromatographic variable used to suppress analyte ionisation and hence reduce detrimental secondary interactions. This approach was applied to the analysis of ephedrines for doping control analysis,

where good retention and peak shape are required for resolution of the diastereoisomeric pairs and accurate quantification.

Volatile buffer systems, such as ammonium formate and ammonium bicarbonate, enable the manipulation of the ionisation state of an analyte in order to produce good chromatography, while maintaining MS operation. Temperature has also been investigated as an important additional variable in LC. The combination of high pH and elevated temperature, now feasible with chemically stable hybrid column materials, is shown to have a significant influence on the peak shape of basic analytes, improving both peak fronting and tailing while also increasing sensitivity and enabling faster analysis. An increase in temperature does not provide an increase in separation efficiency, but renders the use of longer or coupled column formats feasible in generating higher plate numbers as a result of reduced mobile phase viscosity.

Additionally, HILIC has been evaluated as an alternative mode of separation for hydrophilic bases, presenting further advantages over RPLC. Kinetic benefits are obtained from the highly organic mobile phases employed, providing low mobile phase viscosities and better mass transfer characteristics leading to higher efficiency separations. The highly organic mobile phase employed also provides enhanced ESI desolvation resulting in higher sensitivity and lower detection limits, as well as permitting the use of longer columns or increased flow rates without the back-pressure constraints presented by RPLC solvents. For the compounds studied, more symmetrical peak shapes and improved sample loading capacity were noted with HILIC. While linearity, accuracy, precision and matrix effects were similar for both

approaches, HILIC provided a six-fold increase in signal-to-noise and was better able to tolerate high sample masses on column, affording a greater linear dynamic range. However despite these advantages, HILIC has been associated with poor reproducibility, the sources of which have been investigated and overcome to provide a robust and repeatable quantification method. As HILIC evolves and further efforts focus on the elucidation of mechanism of interaction, this technique is fast becoming a widely adopted tool of polar analytes. There has recently been a surge of interest in this field, and this is reflected by the variety of stationary phase chemistries now available for HILIC, which were previously limited to unbonded silica supports. To further the diversity which HILIC can offer, future work should also consider alternative solvent systems for added selectivity and a more environmentally friendly mobile phase.

An important factor in the sensitivity of a LC-MS method, in addition to the inherent sensitivity of the mass spectrometer, is the efficiency of ionisation and transmission. Although the chemical and physical properties of an analyte have a major influence on ionisation, the use of mobile phase additives and optimisation of source parameters also play a substantial role in ionisation efficiency. The importance of selecting a solvent system for optimal chromatography and MS performance has been described, as well as the influence of source conditions which can be mobile phase and analyte specific. Converse to conventional theory, high pH eluents have been show to elicit up to a three-fold increase in MS response for basic analytes. In addition, with ESI basic analytes have been shown to ionise more effectively using low voltages (0.5 kV).

Sample pre-treatment is especially important for the analysis of complex matrices such as biofluids, where co-eluting endogenous components compete with the analyte for charge in the ion source, possibly resulting in ion suppression or enhancement. The result of ion suppression may be reduced through the use of UHPLC technology, offering better chromatographic resolution of analytes of interest from any endogenous species. Since sample preparation is a major bottleneck with the high-throughput analysis now available, there is even more focus on minimal sample pre-treatment, such as the direct dilution and injection of urine which is presented in this study, and therefore the importance of evaluating matrix effects is paramount. The use of high pH RPLC or HILIC serves to enhance the retention of hydrophilic basic analytes, separating them from the noisy solvent front. However, the extent of ion suppression or enhancement should always be evaluated for a robust and accurate assay.

Although high pH RPLC and HILIC illustrate several advantages for the analysis of hydrophilic and/or basic analytes, where large peak capacities or a combination of different materials are required for the analysis of a diverse range of compounds, these modes cannot easily be coupled with traditional RPLC systems for column switching or 2D LC approaches. Such techniques using these modes of LC require an in-line dilution because of the differing strength solvent systems, which reduces sensitivity. Therefore, polymeric phases have been evaluated for their potential as platforms for the analysis of very polar analytes operated under traditional acidic RPLC conditions. Hypercarb has proven to be a successful material for such a purpose, and permits a facile means of coupling the separation of very polar analytes with RPLC to extend the polarity window for chromatographic analysis. A column

switching system has been designed to enable the separation of a wide range of polarities in a single injection. Although this proof-of-principle design was performed coupled with a UV detector, future work would focus on coupling this configuration with MS. The mobile phase system was designed to enable MS operation, providing a useful approach for the separation and unequivocal identification of samples comprising a wide range of components, such as generic or untargeted screening approaches, or where highly polar metabolites are to be detected in the presence of more non-polar parent compounds. Polymeric stationary phase materials illustrate good retention characteristics towards very polar analytes, although the efficiency of these separations is poor, mainly as a result of poor pressure stability which leads to unstable packing beds. The development of additional alternative stationary phase materials for very polar analytes which can operate under RPLC conditions and at UHPLC pressures is therefore necessary.

The recent shift from the use of tandem triple quadrupole mass spectrometry to high resolution accurate mass spectrometry is becoming widely adopted, especially in areas such as generic screening where high-throughput separations demand UHPLC coupled with fast scanning MS detection. As illustrated in this thesis, with advances in technology there have been significant improvements in the resolution, mass accuracy, sensitivity and linear dynamic range of such instrumentation, permitting quantification as well as qualitative analysis by platforms such as hybrid QTOF MS detectors. Such equipment offers medium resolution and acceptable mass accuracy at a reasonable cost, which when coupled to UHPLC separation permits fast data acquisition for good definition of very narrow peaks generated with sub-2 µm

particles. With growing popularity and acceptance, HRAMS will become a valuable tool for screening applications and quantification in the routine setting.

REFERENCES

1. MacNair JE, Lewis KC, and Jorgenson JW. *Ultrahigh-Pressure Reversed-Phase Liquid Chromatography in Packed Capillary Columns.* Analytical Chemistry, 1997. **69**(6): p. 983-989.
2. MacNair JE, Patel KD, and Jorgenson JW. *Ultrahigh-Pressure Reversed-Phase Capillary Liquid Chromatography: Isocratic and Gradient Elution Using Columns Packed with 1.0-μm Particles.* Analytical Chemistry, 1999. **71**(3): p. 700-708.
3. Mellors JS and Jorgenson JW. *Use of 1.5-μm Porous Ethyl-Bridged Hybrid Particles as a Stationary-Phase Support for Reversed-Phase Ultrahigh-Pressure Liquid Chromatography.* Analytical Chemistry, 2004. **76**(18): p. 5441-5450.
4. Mazzeo JR, D.Neue U, Kele M, and Plumb RS. *A new separation technique takes advantage of sub-2 μm porous particles* Analytical Chemistry, 2005. **77**(23): p. 460 A-467 A.
5. Fountain KJ, Neue UD, Grumbach ES, and Diehl DM. *Effects of extra-column band spreading, liquid chromatography system operating pressure, and column temperature on the performance of sub-2-[mu]m porous particles.* Journal of Chromatography A, 2009. **1216**(32): p. 5979-5988.
6. Thompson JD and Carr PW. *High-Speed Liquid Chromatography by Simultaneous Optimization of Temperature and Eluent Composition.* Analytical Chemistry, 2002. **74**(16): p. 4150-4159.
7. Lestremau F, Cooper A, Szucs R, David F, and Sandra P. *High-efficiency liquid chromatography on conventional columns and instrumentation by using temperature as a variable: I. Experiments with 25 cm × 4.6 mm I.D., 5 [mu]m ODS columns.* Journal of Chromatography A, 2006. **1109**(2): p. 191-196.
8. Cabrera K. *Applications of silica-based monolithic HPLC columns.* Journal of Separation Science, 2004. **27**(10-11): p. 843-852.
9. Volmer DA, Brombacher S, and Whitehead B. *Studies on azaspiracid biotoxins. I. Ultrafast high-resolution liquid chromatography/mass spectrometry separations using monolithic columns.* Rapid Communications in Mass Spectrometry, 2002. **16**(24): p. 2298-2305.
10. Oláh E, Fekete S, Fekete J, and Ganzler K. *Comparative study of new shell-type, sub-2 μm fully porous and monolith stationary phases, focusing on mass-transfer resistance.* Journal of Chromatography A, 2010. **1217**(23): p. 3642-3653.
11. Kirkland JJ, Truszkowski FA, Dilks Jr CH, and Engel GS. *Superficially porous silica microspheres for fast high-performance liquid chromatography of macromolecules.* Journal of Chromatography A, 2000. **890**(1): p. 3-13.
12. Cunliffe JM and Maloney TD. *Fused-core particle technology as an alternative to sub-2-μm particles to achieve high separation efficiency with low backpressure.* Journal of Separation Science, 2007. **30**: p. 3104-3109.
13. Gritti F and Guiochon G. *Comparative study of the performance of columns packed with several new fine silica particles: Would the external roughness of the particles affect column properties?* Journal of Chromatography A, 2007. **1166**(1–2): p. 30-46.

14. Zhang Y, Wang X, Mukherjee P, and Petersson P. *Critical comparison of performances of superficially porous particles and sub-2 [mu]m particles under optimized ultra-high pressure conditions.* Journal of Chromatography A, 2009. **1216**(21): p. 4597-4605.
15. Nguyen DTT, Guillarme D, Rudaz S, and Veuthey J-L. *Chromatographic behaviour and comparison of column packed with sub-2 µm stationary phases in liquid chromatography.* Journal of Chromatography A, 2006. **1128**(1–2): p. 105-113.
16. de Villiers A, Lestremau F, Szucs R, Gélébart S, David F, and Sandra P. *Evaluation of ultra performance liquid chromatography: Part I. Possibilities and limitations.* Journal of Chromatography A, 2006. **1127**(1-2): p. 60-69.
17. Churchwell MI, Twaddle NC, Meeker LR, and Doerge DR. *Improving LC-MS sensitivity through increases in chromatographic performance: Comparisons of UPLC-ES/MS/MS to HPLC-ES/MS/MS.* Journal of Chromatography B, 2005. **825**(2): p. 134-143.
18. Guillarme D, Nguyen DTT, Rudaz S, and Veuthey J-L. *Method transfer for fast liquid chromatography in pharmaceutical analysis: Application to short columns packed with small particle. Part I: Isocratic separation.* European Journal of Pharmaceutics and Biopharmaceutics, 2007. **66**(3): p. 475-482.
19. Nováková L, Matysová L, and Solich P. *Advantages of application of UPLC in pharmaceutical analysis.* Talanta, 2006. **68**(3): p. 908-918.
20. Guillarme D, Schappler J, Rudaz S, and Veuthey J-L. *Coupling ultra-high-pressure liquid chromatography with mass spectrometry.* TrAC Trends in Analytical Chemistry, 2010. **29**(1): p. 15-27.
21. Holčapek M, Jirásko R, and Lísa M. *Recent developments in liquid chromatography–mass spectrometry and related techniques.* Journal of Chromatography A, 2012. **1259**(0): p. 3-15.
22. Ramanathan R, Jemal M, Ramagiri S, Xia Y-Q, Humpreys WG, Olah T, and Korfmacher WA. *It is time for a paradigm shift in drug discovery bioanalysis: from SRM to HRMS.* Journal of Mass Spectrometry, 2011. **46**(6): p. 595-601.
23. Kaufmann A, Butcher P, Maden K, and Widmer M. *Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-[mu]m particulate high-performance liquid chromatography coupled to time of flight mass spectrometry.* Journal of Chromatography A, 2008. **1194**(1): p. 66-79.
24. Badoud F, Grata E, Perrenoud L, Saugy M, Rudaz S, and Veuthey JL. *Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry. II: Confirmatory analysis.* Journal of Chromatography A, 2010. **1217**(25): p. 4109-4119.
25. Vonaparti A, Lyris E, Angelis YS, Panderi I, Koupparis M, Tsantili-Kakoulidou A, Peters RJB, Nielen MWF, and Georgakopoulos C. *Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry.* Rapid Communications in Mass Spectrometry, 2010. **24**(11): p. 1595-1609.
26. van der Heeft E, Bolck YJC, Beumer B, Nijrolder AWJM, Stolker AAM, and Nielen MWF. *Full-Scan Accurate Mass Selectivity of Ultra-Performance Liquid Chromatography Combined with Time-of-Flight and Orbitrap Mass*

Spectrometry in Hormone and Veterinary Drug Residue Analysis. Journal of the American Society for Mass Spectrometry, 2009. **20**(3): p. 451-463.
27. Touber ME, van Engelen MC, Georgakopoulus C, van Rhijn JA, and Nielen MWF. *Multi-detection of corticosteroids in sports doping and veterinary control using high-resolution liquid chromatography/time-of-flight mass spectrometry.* Analytica Chimica Acta, 2007. **586**(1-2): p. 137-146.
28. Kolmonen M, Leinonen A, Kuuranne T, Pelander A, and Ojanperä I. *Generic sample preparation and dual polarity liquid chromatography - time-of-flight mass spectrometry for high-throughput screening in doping analysis.* Drug Testing and Analysis, 2009. **1**(6): p. 250-266.
29. Thieme D, Grosse R, Lang R, Mueller RK, and Wahl A. *Screening, confirmation and quantification of diuretics in urine for doping control analysis by high-performance liquid chromatography-atmospheric pressure ionisation tandem mass spectrometry.* Journal of Chromatography B, 2001. **757**: p. 49-57.
30. Thevis M, Opfermann G, and Schänzer W. *High speed determination of beta-receptor blocking agents in human urine by liquid chromatography/tandem mass spectrometry.* Biomedical Chromatography, 2001. **15**(6): p. 393-402.
31. Thörngren J-O, Östervall F, and Garle M. *A high-throughput multicomponent screening method for diuretics, masking agents, central nervous system (CNS) stimulants and opiates in human urine by UPLC-MS/MS.* Journal of Mass Spectrometry, 2008. **43**(7): p. 980-992.
32. Mazzarino M, de la Torre X, and Botrè F. *A screening method for the simultaneous detection of glucocorticoids, diuretics, stimulants, anti-oestrogens, beta-adrenergic drugs and anabolic steroids in human urine by LC-ESI-MS/MS.* Analytical and Bioanalytical Chemistry, 2008. **392**(4): p. 681-698.
33. Mazzarino M, de la Torre X, Botrè F, Gray N, and Cowan D. *A rapid screening LC-MS/MS method based on conventional HPLC pumps for the analysis of low molecular weight xenobiotics: application to doping control analysis.* Drug Testing and Analysis, 2010. **2**(7): p. 311-322.
34. IUPAC. *Compendium of Analytical Nomenclature Definitve Rules 1997*http://old.iupac.org/publications/analytical_compendium/.
35. Neue U. *HPLC Columns Theory, Technology and Practice*1997: Wiley
36. Dong MW. *Modern HPLC for Practicing Scientists*2006: Wiley.
37. Dolan JW. *Extracolumn Effects.* LCGC Europe, 2005. **18**(3): p. 130-136.
38. Giddings JC. *Comparison of Theoretical Limit of Separating Speed in Gas and Liquid Chromatography.* Analytical Chemistry, 1965. **37**(1): p. 60-63.
39. Neue UD. *Kinetic Plots Made Easy.* LCGC North America, 2009.
40. Poppe H. *Some reflections on speed and efficiency of modern chromatographic methods.* Journal of Chromatography A, 1997. **778**(1–2): p. 3-21.
41. Bristow P and Knox J. *Standardization of test conditions for high performance liquid chromatography columns.* Chromatographia, 1977. **10**(6): p. 279-289.
42. Desmet G, Clicq D, and Gzil P. *Geometry-independent plate height representation methods for the direct comparison of the kinetic performance of LC supports with a different size or morphology.* Anal Chem, 2005. **77**(13): p. 4058-70.

43. Strege MA, Stevenson S, and Lawrence SM. *Mixed-Mode Anion−Cation Exchange/Hydrophilic Interaction Liquid Chromatography−Electrospray Mass Spectrometry as an Alternative to Reversed Phase for Small Molecule Drug Discovery.* Analytical Chemistry, 2000. **72**(19): p. 4629-4633.
44. Terfloth G. *Enantioseparations in super- and subcritical fluid chromatography.* Journal of Chromatography A, 2001. **906**(1–2): p. 301-307.
45. Snyder LR, Kirkland JJ, and Dolan JW. *Introduction to modern liquid chromatography*. 3rd ed2010: John Wiley & Sons Inc.
46. Neue UD, Tran K, Méndez A, and Carr PW. *The combined effect of silanols and the reversed-phase ligand on the retention of positively charged analytes.* Journal of Chromatography A, 2005. **1063**(1-2): p. 35-45.
47. McCalley DV. *Comparison of peak shapes obtained with volatile (mass spectrometry-compatible) buffers and conventional buffers in reversed-phase high-performance liquid chromatography of bases on particulate and monolithic columns.* Journal of Chromatography A, 2003. **987**(1-2): p. 17-28.
48. McCalley DV. *The challenges of the analysis of basic compounds by high performance liquid chromatography: Some possible approaches for improved separations.* Journal of Chromatography A, 2010. **1217**(6): p. 858-880.
49. Hanai T. *Separation of polar compounds using carbon columns.* Journal of Chromatography A, 2003. **989**(2): p. 183-196.
50. Knox JH and Ross P. *Carbon-based packing materials for liquid chromatography.* Advances in Chromatography, 1997. **37**: p. 73.
51. Wyndham KD, O'Gara JE, Walter TH, Glose KH, Lawrence NL, Alden BA, Izzo GS, Hudalla CJ, and Iraneta PC. *Characterization and Evaluation of C18 HPLC Stationary Phases Based on Ethyl-Bridged Hybrid Organic/Inorganic Particles.* Analytical Chemistry, 2003. **75**(24): p. 6781-6788.
52. Méndez A, Bosch E, Rosés M, and Neue UD. *Comparison of the acidity of residual silanol groups in several liquid chromatography columns.* Journal of Chromatography A, 2003. **986**(1): p. 33-44.
53. Nawrocki J. *The silanol group and its role in liquid chromatography.* J Chromatogr A, 1997. **779**(1-2): p. 29-71.
54. Kirkland JJ, van Straten MA, and Claessens HA. *High pH mobile phase effects on silica-based reversed-phase high-performance liquid chromatographic columns.* Journal of Chromatography A, 1995. **691**(1-2): p. 3-19.
55. Wyndham KD, Walter TH, Iraneta PC, Uwe ND, McDonald PD, Morrison D, and Baynham M. *A review of Waters hybrid particle technology. Part 2. Ethylene- bridged [BEH technology] hybrids and their use in liquid chromatography*, in *Waters Corporation*2004: Massachusetts.
56. Gritti F, Perdu C, and Guiochon G. *Comparison of the performance of a few packing materials designed to minimize the thermodynamic band tailing of basic compounds in reversed-phase liquid chromatography.* Journal of Chromatography A, 2008. **1180**(1-2): p. 73-89.
57. Wyndham KD, Walter TH, Iraneta P, Neue U, McDonald JD, Morrison D, and Baynham M. *http://www.waters.com/webassets/cms/library/docs/720001159en.pdf*. 2004.

58. Alpert AJ. *Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds.* Journal of Chromatography, 1990. **499**: p. 177-196.
59. McCalley DV and Neue UD. *Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography.* Journal of Chromatography A, 2008. **1192**(2): p. 225-229.
60. McCalley DV. *Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography.* Journal of Chromatography A, 2010. **1217**(20): p. 3408-3417.
61. *Waters Corporation.* http://www.waters.com/waters/nav.htm?cid=513211.
62. Neue U, Cheng Y, Lu Z, Alden B, Iraneta P, Khoebe C, and Van Tran K. *Properties of reversed phase packings with an embedded polar group.* Chromatographia, 2001. **54**(3): p. 169-177.
63. Tate PA and Dorsey JG. *Column selection for liquid chromatographic estimation of the kw' hydrophobicity parameter.* Journal of Chromatography A, 2004. **1042**(1-2): p. 37-48.
64. McCalley DV. *Effect of temperature and flow-rate on analysis of basic compounds in high-performance liquid chromatography using a reversed-phase column.* Journal of Chromatography A, 2000. **902**(2): p. 311-321.
65. Buckenmaier SMC, McCalley DV, and Euerby MR. *Rationalisation of unusual changes in efficiency and retention with temperature shown for bases in reversed-phase high-performance liquid chromatography at intermediate pH.* Journal of Chromatography A, 2004. **1060**(1-2): p. 117-126.
66. Guillarme D, Heinisch S, and Rocca JL. *Effect of temperature in reversed phase liquid chromatography.* Journal of Chromatography A, 2004. **1052**(1–2): p. 39-51.
67. Teutenberg T, Hollebekkers K, Wiese S, and Boergers A. *Temperature and pH-stability of commercial stationary phases.* Journal of Separation Science, 2009. **32**(9): p. 1262-1274.
68. Plumb R, Mazzeo JR, Grumbach ES, Rainville P, Jones M, Wheat T, Neue UD, Smith B, and Johnson KA. *The application of small porous particles, high temperatures, and high pressures to generate very high resolution LC and LC/MS separations.* Journal of Separation Science, 2007. **30**(8): p. 1158-1166.
69. Fenn JB, Mann M, Meng CK, Wong SF, and Whitehouse CM. *Electrospray ionization–principles and practice.* Mass Spectrometry Reviews, 1990. **9**(1): p. 37-70.
70. Dole M, Mack LL, and Hines RL. *Molecular beams of macroions.* Journal of Chemical Physics, 1968. **49**(5): p. 2240-&.
71. Iribarne JV and Thomson BA. *Evaporation of small ions from charged droplets* Journal of Chemical Physics, 1976. **64**(6): p. 2287-2294.
72. Thomson BA and Iribarne JV. *Field- induced evaporation from liquid surfaces at atmospheric pressure.* Journal of Chemical Physics, 1979. **71**(11): p. 4451-4463.
73. *http://www.bris.ac.uk/nerclsmsf/images/apci2.gif.* University of Bristol.
74. *http://www.chm.bris.ac.uk/ms/theory/quad-massspec.html.* University of Bristol.

75. Zhang N, Fountain ST, Bi H, and Rossi DT. *Quantification and Rapid Metabolite Identification in Drug Discovery Using API Time-of-Flight LC/MS.* Analytical Chemistry, 2000. **72**(4): p. 800-806.
76. Wrona M, Mauriala T, Bateman KP, Mortishire-Smith RJ, and O'Connor D. *'All-in-One' analysis for metabolite identification using liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry with collision energy switching.* Rapid Communications in Mass Spectrometry, 2005. **19**(18): p. 2597-2602.
77. Broecker S, Herre S, and Pragst F. *General unknown screening in hair by liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC–QTOF-MS).* Forensic Science International, 2012. **218**(1–3): p. 68-81.
78. *http://www.whri.qmul.ac.uk/PAU/MSunit_QTOF.html.* Queen Mary University of London
79. Dams R, Benijts T, Gunther W, Lambert W, and De Leenheer A. *Influence of the eluent composition on the ionization efficiency for morphine of pneumatically assisted electrospray, atmospheric-pressure chemical ionization and sonic spray.* Rapid Communications in Mass Spectrometry, 2002. **16**(11): p. 1072-1077.
80. Cech NB and Enke CG. *Practical implications of some recent studies in electrospray ionization fundamentals.* Mass Spectrometry Reviews, 2001. **20**(6): p. 362-387.
81. Kostiainen R and Kauppila TJ. *Effect of eluent on the ionization process in liquid chromatography-mass spectrometry.* Journal of Chromatography A, 2009. **1216**(4): p. 685-699.
82. Peng L and Farkas T. *Analysis of basic compounds by reversed-phase liquid chromatography-electrospray mass spectrometry in high-pH mobile phases.* Journal of Chromatography A, 2008. **1179**(2): p. 131-144.
83. Apffel A, Fischer S, Goldberg G, Goodley PC, and Kuhlmann FE. *Enhanced sensitivity for peptide mapping with electrospray liquid chromatography-mass spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases.* Journal of Chromatography A, 1995. **712**(1): p. 177-190.
84. Wang G and Cole RB. *Effects of solvent and counterion on ion pairing and observed charge states of diquaternary ammonium salts in electrospray ionization mass spectrometry.* Journal of the American Society for Mass Spectrometry, 1996. **7**(10): p. 1050-1058.
85. Kuhlmann FE, Apffel A, Fischer SM, Goldberg G, and Goodley PC. *Signal enhancement for gradient reverse-phase high-performance liquid chromatography-electrospray ionization mass spectrometry analysis with trifluoroacetic and other strong acid modifiers by postcolumn addition of propionic acid and isopropanol.* Journal of the American Society for Mass Spectrometry, 1995. **6**(12): p. 1221-1225.
86. Dams R, Huestis MA, Lambert WE, and Murphy CM. *Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid.* Journal of the American Society for Mass Spectrometry, 2003. **14**(11): p. 1290-1294.
87. Deventer K, Pozo OJ, Van Eenoo P, and Delbeke FT. *Qualitative detection of diuretics and acidic metabolites of other doping agents in human urine by high-performance liquid chromatography-tandem mass spectrometry:*

Comparison between liquid-liquid extraction and direct injection. Journal of Chromatography A, 2009. **1216**(31): p. 5819-5827.

88. Van De Steene JC and Lambert WE. *Comparison of Matrix Effects in HPLC-MS/MS and UPLC-MS/MS Analysis of Nine Basic Pharmaceuticals in Surface Waters.* Journal of the American Society for Mass Spectrometry, 2008. **19**(5): p. 713-718.
89. Avois L, Robinson N, Saudan C, Baume N, Mangin P, and Saugy M. *Central nervous system stimulants and sport practice.* British Journal of Sports Medicine, 2006. **40**(suppl 1): p. i16-i20.
90. The World Anti-Doping Code: The 2012 Prohibited List International Standard.
91. Gmeiner G, Geisendorfer T, Kainzbauer J, Nikolajevic M, and Tausch H. *Quantification of ephedrines in urine by column-switching high-performance liquid chromatography.* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2002. **768**(2): p. 215-221.
92. Spyridaki M-HE, Tsitsimpikou CJ, Siskos PA, and Georgakopoulos CG. *Determination of ephedrines in urine by gas chromatography–mass spectrometry.* Journal of Chromatography B: Biomedical Sciences and Applications, 2001. **758**(2): p. 311-314.
93. Kolmonen M, Leinonen A, Pelander A, and Ojanperä I. *A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry.* Analytica Chimica Acta, 2007. **585**(1): p. 94-102.
94. Spyridaki MH, Kiousi P, Vonaparti A, Valavani P, Zonaras V, Zahariou M, Sianos E, Tsoupras G, and Georgakopoulos C. *Doping control analysis in human urine by liquid chromatography-electrospray ionization ion trap mass spectrometry for the Olympic Games Athens 2004: Determination of corticosteroids and quantification of ephedrines, salbutamol and morphine.* Analytica Chimica Acta, 2006. **573-574**: p. 242-249.
95. Badoud F, Grata E, Perrenoud L, Avois L, Saugy M, Rudaz S, and Veuthey JL. *Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry: I. Screening analysis.* Journal of Chromatography A, 2009. **1216**(20): p. 4423-4433.
96. Deventer K, Pozo OJ, Van Eenoo P, and Delbeke FT. *Development and validation of an LC-MS/MS method for the quantification of ephedrines in urine.* Journal of Chromatography B, 2009. **877**(4): p. 369-374.
97. Thevis M and Schänzer W. *Examples of doping control analysis by liquid chromatography-tandem mass spectrometry: Ephedrines, β-receptor blocking agents, diuretics, sympathomimetics, and cross-linked hemoglobins.* Journal of Chromatographic Science, 2005. **43**(1): p. 22-31.
98. Mallet CR, Lu Z, and Mazzeo JR. *A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts.* Rapid Communications in Mass Spectrometry, 2004. **18**(1): p. 49-58.
99. Gustavsson SÅ, Samskog J, Markides KE, and Långström B. *Studies of signal suppression in liquid chromatography–electrospray ionization mass spectrometry using volatile ion-pairing reagents.* Journal of Chromatography A, 2001. **937**(1–2): p. 41-47.
100. McCalley DV and Brereton RG. *High-performance liquid chromatography of basic compounds: Problems, possible solutions and tests of reversed-phase columns.* Journal of Chromatography A, 1998. **828**(1-2): p. 407-420.

101. Neue UD, Phoebe CH, Tran K, Cheng Y-F, and Lu Z. *Dependence of reversed-phase retention of ionizable analytes on pH, concentration of organic solvent and silanol activity.* Journal of Chromatography A, 2001. **925**(1-2): p. 49-67.
102. Kirkland JJ, A vSM, and A CH. *Reversed-phase high-performance liquid chromatography of basic compounds at pH 11 with silica-based column packings.* Journal of Chromatography A, 1998. **797**: p. 111-120.
103. Kirkland JJ. *Development of some stationary phases for reversed-phase HPLC.* Journal of Chromatography A, 2004. **1060**(1–2): p. 9-21.
104. Davies NH, Euerby MR, and McCalley DV. *Study of overload for basic compounds in reversed-phase high performance liquid chromatography as a function of mobile phase pH.* Journal of Chromatography A, 2006. **1119**(1-2): p. 11-19.
105. McCalley DV. *Rationalization of Retention and Overloading Behavior of Basic Compounds in Reversed-Phase HPLC Using Low Ionic Strength Buffers Suitable for Mass Spectrometric Detection.* Analytical Chemistry, 2003. **75**(14): p. 3404-3410.
106. Neue UD, Wheat TE, Mazzeo JR, Mazza CB, Cavanaugh JY, Xia F, and Diehl DM. *Differences in preparative loadability between the charged and uncharged forms of ionizable compounds.* Journal of Chromatography, 2004. **1030**(1-2): p. 123-134.
107. Davies NH, Euerby MR, and McCalley DV. *Analysis of basic compounds by reversed-phase high-performance liquid chromatography using hybrid inorganic/organic phases at high pH.* Journal of Chromatography A, 2008. **1178**(1-2): p. 71-78.
108. Mansoori BA, Volmer DA, and Boyd RK. *"Wrong-way-round" Electrospray Ionization of Amino Acids.* Rapid Communications in Mass Spectrometry, 1997. **11**(10): p. 1120-1130.
109. Zhou S and Cook KD. *Protonation in electrospray mass spectrometry: wrong-way-round or right-way-round?* Journal of the American Society for Mass Spectrometry, 2000. **11**(11): p. 961-966.
110. Rainville PD, Smith NW, Cowan D, and Plumb RS. *Comprehensive investigation of the influence of acidic, basic, and organic mobile phase compositions on bioanalytical assay sensitivity in positive ESI mode LC/MS/MS.* Journal of Pharmaceutical and Biomedical Analysis, 2012. **59**(0): p. 138-150.
111. Dolan JW. *Temperature selectivity in reversed-phase high performance liquid chromatography.* Journal of Chromatography A, 2002. **965**(1–2): p. 195-205.
112. Lestremau F, de Villiers A, Lynen F, Cooper A, Szucs R, and Sandra P. *High efficiency liquid chromatography on conventional columns and instrumentation by using temperature as a variable: Kinetic plots and experimental verification.* Journal of Chromatography A, 2007. **1138**(1–2): p. 120-131.
113. McNeff CV, Yan B, Stoll DR, and Henry RA. *Practice and theory of high temperature liquid chromatography.* Journal of Separation Science, 2007. **30**(11): p. 1672-1685.
114. Sandra K, Verleysen K, Labeur C, Vanneste L, D'Hondt F, Thomas G, Kas K, Gevaert K, Vandekerckhove J, and Sandra P. *Combination of COFRADIC and high temperature – extended column length conventional liquid*

chromatography: A very efficient way to tackle complex protein samples, such as serum. Journal of Separation Science, 2007. **30**(5): p. 658-668.
115. Wilke CR and Chang P. *Correlation of diffusion coefficients in dilute solutions.* AIChE Journal, 1955. **1**(2): p. 264-270.
116. LoBrutto R, Jones A, Kazakevich YV, and McNair HM. *Effect of the eluent pH and acidic modifiers in high-performance liquid chromatography retention of basic analytes.* Journal of Chromatography A, 2001. **913**(1–2): p. 173-187.
117. Dai J and Carr PW. *Role of ion pairing in anionic additive effects on the separation of cationic drugs in reversed-phase liquid chromatography.* Journal of Chromatography A, 2005. **1072**(2): p. 169-184.
118. Canals I, Portal JA, Bosch E, and Rosés M. *Retention of Ionizable Compounds on HPLC. 4. Mobile-Phase pH Measurement in Methanol/Water.* Analytical Chemistry, 2000. **72**(8): p. 1802-1809.
119. Al-Tannak NF, Bawazeer S, Siddiqui TH, and Watson DG. *The hydrophilic interaction like properties of some reversed phase high performance liquid chromatography columns in the analysis of basic compounds.* Journal of Chromatography A, 2011. **1218**(11): p. 1486-1491.
120. Vanhoenacker G and Sandra P. *Elevated temperature and temperature programming in conventional liquid chromatography – fundamentals and applications.* Journal of Separation Science, 2006. **29**(12): p. 1822-1835.
121. Yan B, Zhao J, Brown JS, Blackwell J, and Carr PW. *High-Temperature Ultrafast Liquid Chromatography* Analytical Chemistry, 2000. **72**: p. 1253-1262.
122. D. Antia F and Horváth C. *High-performance liquid chromatography at elevated temperatures: examination of conditions for the rapid separation of large molecules.* Journal of Chromatography A, 1988. **435**(0): p. 1-15.
123. World Anti-Doping Agency. *The World Anti-Doping Code: The 2010 Prohibited List International Standard.* 2010.
124. Gao S, Zhang Z-P, and Karnes HT. *Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives.* Journal of Chromatography B, 2005. **825**(2): p. 98-110.
125. Rauha J-P, Vuorela H, and Kostiainen R. *Effect of eluent on the ionization efficiency of flavonoids by ion spray, atmospheric pressure chemical ionization, and atmospheric pressure photoionization mass spectrometry.* Journal of Mass Spectrometry, 2001. **36**(12): p. 1269-1280.
126. de Jonge ME, van Dam SM, Hillebrand MJX, Rosing H, Huitema ADR, Rodenhuis S, and Beijnen JH. *Simultaneous quantification of cyclophosphamide, 4-hydroxycyclophosphamide, N,N′,N″-triethylenethiophosphoramide (thiotepa) and N,N′,N″-triethylenephosphoramide (tepa) in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry.* Journal of Mass Spectrometry, 2004. **39**(3): p. 262-271.
127. Stokvis E, Rosing H, Crul M, Rieser MJ, Heck AJR, Schellens JHM, and Beijnen JH. *Quantitative analysis of the novel anticancer drug ABT-518, a matrix metalloproteinase inhibitor, plus the screening of six metabolites in human plasma using high-performance liquid chromatography coupled with electrospray tandem mass spectrometry.* Journal of Mass Spectrometry, 2004. **39**(3): p. 277-288.

128. Vainchtein LD, Rosing H, Mirejovsky D, Huynh V, Lenaz L, Hillebrand MJX, Schellens JHM, and Beijnen JH. *Quantitative analysis of EO9 (apaziquone) and its metabolite EO5a in human plasma by high-performance liquid chromatography under basic conditions coupled to electrospray tandem mass spectrometry.* Journal of Mass Spectrometry, 2006. **41**(10): p. 1268-1276.
129. Cheng YF, Lu Z, and Neue U. *Ultrafast liquid chromatography/ultraviolet and liquid chromatography/tandem mass spectrometric analysis.* Rapid Communications in Mass Spectrometry, 2001. **15**(2): p. 141-151.
130. Amad MaH, Cech NB, Jackson GS, and Enke CG. *Importance of gas-phase proton affinities in determining the electrospray ionization response for analytes and solvents.* Journal of Mass Spectrometry, 2000. **35**(7): p. 784-789.
131. Van Berkel GJ, Zhou F, and Aronson JT. *Changes in bulk solution pH caused by the inherent controlled-current electrolytic process of an electrospray ion source.* International Journal of Mass Spectrometry and Ion Processes, 1997. **162**(1–3): p. 55-67.
132. Gatlin CL and Turecek F. *Acidity Determination in Droplets Formed by Electrospraying Methanol-Water Solutions.* Analytical Chemistry, 1994. **66**(5): p. 712-718.
133. Sillén H and Magnell N. *Screening ionisation and chromatography conditions for quantitative LC/MS methods.* Journal of Chromatography B, 2009. **877**(29): p. 3581-3588.
134. Straube EA, Dekant W, and Völkel W. *Comparison of electrospray ionization, atmospheric pressure chemical ionization, and atmospheric pressure photoionization for the analysis of dinitropyrene and aminonitropyrene LC-MS/MS.* Journal of the American Society for Mass Spectrometry, 2004. **15**(12): p. 1853-1862.
135. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, and Olah T. *Mechanistic investigation of ionization suppression in electrospray ionization.* Journal of the American Society for Mass Spectrometry, 2000. **11**(11): p. 942-950.
136. Matuszewski BK. *Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis.* Journal of Chromatography B, 2006. **830**(2): p. 293-300.
137. World Anti-Doping Agency. *WADA Technical Document TD2003IDCR*. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2003IDCR_EN.pdf 2003.
138. Flieger J. *Application of perfluorinated acids as ion-pairing reagents for reversed-phase chromatography and retention-hydrophobicity relationships studies of selected [beta]-blockers.* Journal of Chromatography A, 2010. **1217**(4): p. 540-549.
139. Ganzera M, Lanser C, and Stuppner H. *Simultaneous determination of Ephedra sinica and Citrus aurantium var. amara alkaloids by ion-pair chromatography.* Talanta, 2005. **66**(4): p. 889-894.
140. Gray N, Musenga A, Cowan DA, Plumb R, and Smith NW. *A simple high pH liquid chromatography–tandem mass spectrometry method for basic compounds: Application to ephedrines in doping control analysis.* Journal of Chromatography A, 2011. **1218**(15): p. 2098-2105.

141. McCalley DV. *Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds?* Journal of Chromatography A, 2007. **1171**(1-2): p. 46-55.
142. Aturki Z, D'Orazio G, Rocco A, Si-Ahmed K, and Fanali S. *Investigation of polar stationary phases for the separation of sympathomimetic drugs with nano-liquid chromatography in hydrophilic interaction liquid chromatography mode.* Analytica Chimica Acta, 2011. **685**(1): p. 103-110.
143. Kolmonen M, Leinonen A, Kuuranne T, Pelander A, and Ojanperä I. *Hydrophilic interaction liquid chromatography and accurate mass measurement for quantification and confirmation of morphine, codeine and their glucuronide conjugates in human urine.* Journal of Chromatography B, 2010. **878**(29): p. 2959-2966.
144. Marclay F and Saugy M. *Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography–tandem mass spectrometry: Potential use of smokeless tobacco products by ice hockey players.* Journal of Chromatography A, 2010. **1217**(48): p. 7528-7538.
145. Mazzarino M, Fiacco I, de la Torre X, and Botrè F. *Screening and confirmation analysis of stimulants, narcotics and beta-adrenergic agents in human urine by hydrophilic interaction liquid chromatography coupled to mass spectrometry.* Journal of Chromatography A, 2011. **1218**(45): p. 8156-8167.
146. Mitchell CR, Bao Y, Benz NJ, and Zhang S. *Comparison of the sensitivity of evaporative universal detectors and LC/MS in the HILIC and the reversed-phase HPLC modes.* Journal of Chromatography B, 2009. **877**(32): p. 4133-4139.
147. Appelblad P, Jonsson T, Jiang W, and Irgum K. *Fast hydrophilic interaction liquid chromatographic separations on bonded zwitterionic stationary phase.* Journal of Separation Science, 2008. **31**(9): p. 1529-1536.
148. Heaton J, Gray N, Cowan DA, Plumb RS, Legido-Quigley C, and Smith NW. *Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the separation of ephedrines.* Journal of Chromatography A, 2012. **1228**(0): p. 329-337.
149. Hemström P and Irgum K. *Hydrophilic interaction chromatography.* Journal of Separation Science, 2006. **29**(12): p. 1784-1821.
150. Karatapanis AE, Fiamegos YC, and Stalikas CD. *A revisit to the retention mechanism of hydrophilic interaction liquid chromatography using model organic compounds.* J Chromatogr A, 2011. **1218**(20): p. 2871-2879.
151. Kumar A, Hart JP, and McCalley DV. *Determination of catecholamines in urine using hydrophilic interaction chromatography with electrochemical detection.* Journal of Chromatography A, 2011. **1218**(25): p. 3854-3861.
152. Ojanperä I, Kolmonen M, and Pelander A. *Current use of high-resolution mass spectrometry in drug screening relevant to clinical and forensic toxicology and doping control.* Analytical and Bioanalytical Chemistry, 2012. **403**(5): p. 1203-1220.
153. Kaufmann A, Butcher P, Maden K, Walker S, and Widmer M. *Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry.* Rapid Communications in Mass Spectrometry, 2011. **25**(7): p. 979-992.

154. Peters R, Oosterink J, Stolker A, Georgakopoulos C, and Nielen M. *Generic sample preparation combined with high-resolution liquid chromatography–time-of-flight mass spectrometry for unification of urine screening in doping-control laboratories.* Analytical and Bioanalytical Chemistry, 2010. **396**(7): p. 2583-2598.
155. Badoud F, Grata E, Boccard J, Guillarme D, Veuthey J-L, Rudaz S, and Saugy M. *Quantification of glucuronidated and sulfated steroids in human urine by ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry.* Analytical and Bioanalytical Chemistry, 2011. **400**(2): p. 503-516.
156. Danaceau JP, Scott Morrison M, and Slawson MH. *Quantitative confirmation of testosterone and epitestosterone in human urine by LC/Q-ToF mass spectrometry for doping control.* Journal of Mass Spectrometry, 2008. **43**(7): p. 993-1000.
157. Spagou K, Wilson ID, Masson P, Theodoridis G, Raikos N, Coen M, Holmes E, Lindon JC, Plumb RS, Nicholson JK, and Want EJ. *HILIC-UPLC-MS for Exploratory Urinary Metabolic Profiling in Toxicological Studies.* Analytical Chemistry, 2010. **83**(1): p. 382-390.
158. Miller Iv JH, Poston PA, and Karnes HT. *A quantitative method for acylcarnitines and amino acids using high resolution chromatography and tandem mass spectrometry in newborn screening dried blood spot analysis.* Journal of Chromatography B, 2012. **903**(0): p. 142-149.
159. Chen P, Li W, Li Q, Wang Y, Li Z, Ni Y, and Koike K. *Identification and quantification of nucleosides and nucleobases in Geosaurus and Leech by hydrophilic-interaction chromatography.* Talanta, 2011. **85**(3): p. 1634-1641.
160. Köhne AP and Welsch T. *Coupling of a microbore column with a column packed with non-porous particles for fast comprehensive two-dimensional high-performance liquid chromatography.* Journal of Chromatography A, 1999. **845**(1–2): p. 463-469.
161. Hu L, Chen X, Kong L, Su X, Ye M, and Zou H. *Improved performance of comprehensive two-dimensional HPLC separation of traditional Chinese medicines by using a silica monolithic column and normalization of peak heights.* Journal of Chromatography A, 2005. **1092**(2): p. 191-198.
162. Chen X, Kong L, Su X, Fu H, Ni J, Zhao R, and Zou H. *Separation and identification of compounds in Rhizoma chuanxiong by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry.* Journal of Chromatography A, 2004. **1040**(2): p. 169-178.
163. Gilar M, Olivova P, Daly AE, and Gebler JC. *Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions.* Journal of Separation Science, 2005. **28**(14): p. 1694-1703.
164. François I, Cabooter D, Sandra K, Lynen F, Desmet G, and Sandra P. *Tryptic digest analysis by comprehensive reversed phase×two reversed phase liquid chromatography (RP-LC×2RP-LC) at different pH's.* Journal of Separation Science, 2009. **32**(8): p. 1137-1144.
165. Louw S, Pereira AS, Lynen F, Hanna-Brown M, and Sandra P. *Serial coupling of reversed-phase and hydrophilic interaction liquid chromatography to broaden the elution window for the analysis of pharmaceutical compounds.* Journal of Chromatography A, 2008. **1208**(1-2): p. 90-94.

166. Kivilompolo M and Hyötyläinen T. *Comparison of separation power of ultra performance liquid chromatography and comprehensive two-dimensional liquid chromatography in the separation of phenolic compounds in beverages.* Journal of Separation Science, 2008. **31**(19): p. 3466-3472.
167. Huidobro AL, Pruim P, Schoenmakers P, and Barbas C. *Ultra rapid liquid chromatography as second dimension in a comprehensive two-dimensional method for the screening of pharmaceutical samples in stability and stress studies.* Journal of Chromatography A, 2008. **1190**(1-2): p. 182-190.
168. Cacciola F, Delmonte P, Jaworska K, Dugo P, Mondello L, and Rader JI. *Employing ultra high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of Stevia rebaudiana extracts.* Journal of Chromatography A, 2011. **1218**(15): p. 2012-2018.
169. Cacciola F, Donato P, Giuffrida D, Torre G, Dugo P, and Mondello L. *Ultra high pressure in the second dimension of a comprehensive two-dimensional liquid chromatographic system for carotenoid separation in red chili peppers.* Journal of Chromatography A, 2012. **1255**(0): p. 244-251.
170. Alexander AJ and Ma L. *Comprehensive two-dimensional liquid chromatography separations of pharmaceutical samples using dual Fused-Core columns in the 2nd dimension.* Journal of Chromatography A, 2009. **1216**(9): p. 1338-1345.
171. Masqué N, Marcé RM, and Borrull F. *New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water.* TrAC Trends in Analytical Chemistry, 1998. **17**(6): p. 384-394.
172. Hennion MC, Cau-Dit-Coumes C, and Pichon V. *Trace analysis of polar organic pollutants in aqueous samples: Tools for the rapid prediction and optimisation of the solid-phase extraction parameters.* Journal of Chromatography A, 1998. **823**(1–2): p. 147-161.
173. Dumont PJ and Fritz JS. *Effect of resin sulfonation on the retention of polar organic compounds in solid-phase extraction.* Journal of Chromatography A, 1995. **691**(1–2): p. 123-131.
174. Davankov VA, Sychov CS, Ilyin MM, and Sochilina KO. *Hypercrosslinked polystyrene as a novel type of high-performance liquid chromatography column packing material: Mechanisms of retention.* Journal of Chromatography A, 2003. **987**(1–2): p. 67-75.
175. Thiébaut D, Vial J, Michel M, Hennion M-C, and Greibrokk T. *Evaluation of reversed phase columns designed for polar compounds and porous graphitic carbon in "trapping" and separating neurotransmitters.* Journal of Chromatography A, 2006. **1122**(1-2): p. 97-104.
176. Vial J, Hennion M-C, Fernandez-Alba A, and Agüera A. *Use of porous graphitic carbon coupled with mass detection for the analysis of polar phenolic compounds by liquid chromatography.* Journal of Chromatography A, 2001. **937**(1–2): p. 21-29.
177. Barrett D, Pawula M, Knaggs R, and Shaw P. *Retention behavior of morphine and its metabolites on a porous graphitic carbon column.* Chromatographia, 1998. **47**(11): p. 667-672.
178. Melmer M, Stangler T, Premstaller A, and Lindner W. *Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon*

chromatography for glycan analysis. Journal of Chromatography A, 2011. **1218**(1): p. 118-123.
179. Koivisto P, Törnkvist A, Heldin E, and Markides K. *Separation of L-DOPA and four metabolites in plasma using a porous graphitic carbon column in capillary liquid chromatography.* Chromatographia, 2002. **55**(1): p. 39-42.
180. Monser L and Darghouth F. *Simultaneous LC determination of paracetamol and related compounds in pharmaceutical formulations using a carbon-based column.* Journal of Pharmaceutical and Biomedical Analysis, 2002. **27**(6): p. 851-860.
181. Monser L and Darghouth F. *Rapid liquid chromatographic method for simultaneous determination of tetracyclines antibiotics and 6-Epi-doxycycline in pharmaceutical products using porous graphitic carbon column.* Journal of Pharmaceutical and Biomedical Analysis, 2000. **23**(2–3): p. 353-362.
182. Jansen RS, Rosing H, Schellens JHM, and Beijnen JH. *Retention studies of 2'-2'-difluorodeoxycytidine and 2'-2'-difluorodeoxyuridine nucleosides and nucleotides on porous graphitic carbon: Development of a liquid chromatography–tandem mass spectrometry method.* Journal of Chromatography A, 2009. **1216**(15): p. 3168-3174.
183. Gu G and Lim CK. *Separation of anionic and cationic compounds of biomedical interest by high-performance liquid chromatography on porous graphitic carbon.* Journal of Chromatography A, 1990. **515**(0): p. 183-192.
184. Hennion M-C, Coquart V, Guenu S, and Sella C. *Retention behaviour of polar compounds using porous graphitic carbon with water-rich mobile phases.* Journal of Chromatography A, 1995. **712**(2): p. 287-301.
185. Griffiths JR, Perkins S, Connolly Y, Zhang L, Holland M, Barattini V, Pereira L, Edge A, Ritchie H, and Smith DL. *The utility of porous graphitic carbon as a stationary phase in proteomics workflows: Two-dimensional chromatography of complex peptide samples.* Journal of Chromatography A, 2012. **1232**(0): p. 276-280.
186. Clarkson C, Sibum M, Mensen R, and Jaroszewski JW. *Evaluation of on-line solid-phase extraction parameters for hyphenated, high-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance applications.* Journal of Chromatography A, 2007. **1165**(1–2): p. 1-9.

BIBLIOGRAPHY

PUBLICATIONS

A simple high pH liquid chromatography-tandem mass spectrometry method for basic compounds: application to ephedrines in doping control analysis. Gray N, Musenga A, Cowan DA, Plumb RS, Smith NW. Journal of Chromatography A, 2011.**1218**(15): p. 2098-2105.

Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the separation of ephedrines. Heaton J, Gray N, Cowan DA, Plumb RS, Legido-Quigley C, Smith NW. Journal of Chromatography A, 2012. **1228**(0): p. 329-337.

Comparison of reversed-phase and hydrophilic interaction liquid chromatographic for the quantification of ephedrines using high resolution accurate mass spectrometry. Gray N, Heaton J, Musenga A, Cowan DA, Plumb RS, Smith NW. Journal of Chromatography A, 2013. **1289**(0): p. 37-46.

POSTER PRESENTATIONS

Effect of mobile phase pH on the analysis of ephedrine compounds by reverse phase HPLC. Gray N, Cowan DA, Plumb RS, Smith NW. 11th International Symposium on Hyphenated Techniques in Chromatography (HTC-11), January 2010, Bruges, Belgium.

Quantification of Ephedrines in Urine by LC-MS/MS for Doping Control; Comparison of ESI and APCI at High pH. Gray N, Musenga A, Cowan DA, Plumb RS, Smith NW. 36th International Symposium on High Performance Liquid Phase Separations and Related Techniques, June 2010, Boston, MA, USA.

RPLC or HILIC for Quantification of Hydrophilic Bases? Gray N, Heaton J, Musenga A, Cowan DA, Plumb RS, Smith NW. 38th International Symposium on High Performance Liquid Phase Separations and Related Techniques, June 2012, Anaheim, CA, USA.